

Characterisation of the interleukin-4 signalling cascade involved in the induction of cyclooxygenase-2 and the polarisation of macrophages into an anti-inflammatory phenotype

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Abstract

The inflammatory response can be broken down into the pro-inflammatory phase, followed by the resolution phase. Both phases are regulated by the expression or release of immunomodulatory mediators such as cytokines e.g. TNF α and IL-10. The two phases are also associated with different macrophage polarization states where the M1 phenotype is seen during the pro-inflammatory phase and M2 during resolution. Whereas much focus has been on the pro-inflammatory phase, more recently, emphasis has been placed on initiating the resolution phase of inflammation. Historically one of the classical signs of inflammation is the expression of the COX-2 enzyme in neutrophils and macrophages early in the pro-inflammatory phase. However, over the last 20 years, a few research groups have observed a delayed but elevated expression of COX-2 in macrophages, along with a range of cytokines such as IL-10, normally associated with the resolution of inflammation. In addition, other researchers have found that administration of COX-2 inhibitors late in the inflammatory process can exacerbate some chronic inflammatory diseases *in vivo*. The concept of an anti-inflammatory COX-2 was further supported by studies that showed prolonged exposure to diclofenac *in vitro*, induced a COX-2 enzyme and high levels of anti-inflammatory cytokines at a time when resolution would be expected. These observations led to the suggestion that the expression of COX-2 in the resolution phase may in fact be a key mediator of the resolution process. To explore these ideas further, this study focuses on the effects of IL-4, an anti-inflammatory cytokine that is widely associated with the resolution phase of inflammation. A key objective will be to investigate the role of other factors such as peroxisome proliferator-activated receptor gamma (PPAR γ), cyclic adenosine monophosphate (cAMP) and p38 mitogen

activated kinase (MAPK) in the signalling pathways through which IL-4 could potentially induce COX-2 protein and other mediators of resolution.

The experiments were designed to investigate whether there are similarities between the induction of COX-2 and the polarisation of macrophages down an anti-inflammatory line. As such, the macrophage J774.2 cell line was treated with 1µg/ml lipopolysaccharide (LPS), 500µM diclofenac or 10ng/ml IL-4 for 24 and 48 hours. These cells were assessed for COX-2 expression and activity, the production of the pro-inflammatory cytokine, tumour necrosis factor alpha (TNFα) and the anti-inflammatory cytokine, interleukin 10 (IL-10) respectively and the anti-inflammatory mediator, cAMP. The importance of p38 and PPAR_γ was assessed using the specific p38 inhibitor, SB203580 and a PPAR_γ antagonist, Bisphenol A diglycidyl ether (BADGE). Following preliminary pharmacology experiments, the working concentration of each drug was decided: 10µM SB203580 and 150µM BADGE. In a subset of experiments, the effect of inhibition of COX-2 by 0.1-100µM acetaminophen, 1-100µM indomethacin and 1-5µM dexamethasone (DEX) was also investigated; with the aim of identifying differential effects depending on the stimulant.

LPS treated macrophages produced high levels of TNFα and low levels of IL-10 within 24 hours of exposure. By contrast IL-4 produced high IL-10 and low TNFα levels at 48 hours. This confirms the opposing phenotypic state of the macrophages post-stimulation. Following IL-4 stimulation, the studies identified a possible sequence of events leading up to the induction of COX-2 and prostaglandin E₂ (PGE₂) synthesis. Adding antagonists of cAMP, PPAR_γ or p38 MAPK to cells stimulated with IL-4 reduced or blocked COX-2 expression and activity leading to the conclusion that these

factors become activated upstream to COX-2 induction by IL-4. In addition, cAMP was regulated at the level of both PPAR γ and p38 while PPAR γ was regulated by p38. Exposure to indomethacin reduced and blocked TNF α and IL-10 induced by LPS and IL-4 respectively. DEX specifically blocked TNF α secretion but induced IL-10 secretion.

Blocking both p38 and PPAR γ in IL-4 treated cells, prevents secretion of anti-inflammatory cytokine IL-10. The novel finding that the p38 kinase and PPAR γ receptor are required for both the induction of COX-2 and secretion of IL-10, suggests an overlap between macrophage polarisation to an anti-inflammatory phenotype and late COX-2 induction pathways. P38, PPAR γ and cAMP would therefore be reasonable drugs targets to induce resolution. These studies suggest that in addition to being involved in driving inflammation, COX-2 may also drive resolution; however, this is yet to be confirmed with further experiments. These studies may go some way to explaining the adverse reactions and an impaired immune response reported in some individuals administered COX-2 inhibiting NSAIDs.

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Abbreviations

12-HEPE	12-hydroxyeicosatetraenoic acid
AA	Arachidonic acid
AAM	Alternatively activated macrophages
AC	Adenyl cyclase
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ANOVA	Analysis of variance
Arg-1	Arginase-1
BADGE	Bisphenol A diglycidyl ether
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CAM	Classically activated macrophages
cAMP	Cyclic adenosine monophosphate
CEF	Chick embryonic fibroblasts
CHD	Coronary heart disease
COX	Cyclooxygenase
CTFB	Complete transcription factor binding assay buffer
CVD	Cardiovascular disease
cyPG	Cyclopentanone PG
db-cAMP	Dibutyl cyclic adenosine monophosphate
DEX	Dexamethasone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide is an organosulfur
DSS	Dextran sodium sulfate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence

EIA	Enzyme immunoassay
FABP4	Fatty acid binding protein
FBS	Fetal bovine serum
GC	Glucocorticoid
GDP	Guanine diphosphate
GPCR	G protein-coupled receptor
GR	Glucocorticoid receptor
GREs	GC-response elements
GTP	Guanine triphosphate
Gas	G protein alpha subunit
HRP	Horseradish peroxidase
IFN γ	Interferon gamma
Ig	Immunoglobulin
I κ B	Inhibitor of NF κ B
IKK	I κ B kinase
IL	Interleukin
JAK	Janus kinase
KLF4	Kruppel-like factor 4
LHP	Lipid hydroperoxide
LPS	Lipopolysaccharide
MAPKAPK	MAPK-activated protein kinase
MHC	major histocompatibility complex
MK2	MAPKAPK-2
MK3	MAPKAPK-3
MR	Mannose receptor
mRNA	messenger ribonucleic acid
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Non-steroidal anti-inflammatory drugs

NSB	Non-specific binding
p38 MAPK	p38 mitogen activated protein kinase
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PEC	Peritoneal exudate macrophages
PG	Prostaglandin
PGDF	Platelet-derived-growth-factor
PGG ₂	Hydroperoxy endoperoxide prostaglandin G ₂
PGI ₂	Prostacyclin
PKA	Protein kinase A
PLA ₂	Phospholipase A
PMN	Polymorphonuclear
PPAR γ	Peroxisome proliferator-activated receptor gamma
PTGS	Prostaglandin-endoperoxide synthase
PTZ	Pentylentetrazole
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
STAT	Signal transducer and activator of transcription
TGF β	Tumour growth factor-beta
TMB	3,3',5,5'-Tetramethylbenzidine
TNF α	Tumour necrosis factor
UTR	Untranslated region

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Chapter 1: Introduction

1.1 The inflammatory response

Inflammation is a protective host response to physical stress, microorganisms or foreign bodies with the intention of eliminating the noxious stimuli. Discovery of mediators such as cytokines and eicosanoids has improved the current understanding of the complex nature of the immune response. The inflammatory response aims for survival during infection in order to uphold tissue homeostasis (see review: (Kopitar-Jerala, 2017)). Typically, there are 4 parts to the inflammatory response. The signal inducer (bacteria), the sensor (macrophage), the inflammatory mediating molecule (tumour necrosis factor (TNF α)) and the target infected tissue (skin).

There are 5 cardinal signs of inflammation: dolor (pain), rubor (redness), tumor (swelling), calor (heat) and functio laesa (loss of function). Inflammation is characterised by changes in microcirculation, fluid exudation and the movement of leukocytes from blood vessels into tissues (see for reviews: (Lawrence et al., 2002, Maskrey et al., 2011)). Immune cells involved in this process include: neutrophils, monocytes, macrophages, basophils, mast cells, T cells and B cells amongst others (see for review: (Punchard et al., 2004)). The sequential cascade of events control the inflammatory process.

Inflammation is divided into two main processes: the pro-inflammatory phase and the anti-inflammatory phase (resolution) both of which are regulated by immunomodulatory compounds such as cytokines (see for reviews: (Lawrence et al., 2002, Maskrey et al., 2011)). The pro-inflammatory phase encompasses the influx and

accumulation of polymorphonuclear (PMN) leukocytes and chemical mediators such as acute phase proteins. Resolution includes apoptosis of PMN leukocytes, removal of pro-inflammatory mediators and the production of anti-inflammatory mediators and cytokines such as interleukin (IL)-10 (See for reviews: (Lawrence et al., 2002, Osiecki, 2004, Maskrey et al., 2011)). Neutrophil and macrophage production and secretion of cytokines are highly regulated at a transcriptional level and in some cases post-transcriptional level (see review: (Kopitar-Jerala, 2017)).

1.1.1 Acute Inflammation

Acute inflammation is considered the first line of defence and is highly non-specific. There is an orchestration of events including an influx of PMN leukocytes to the site of inflammation, activation of endothelial cells and platelet activation and aggregation. Although previously believed to be a passive process, resolution is now regarded as an active process (see for review: (Serhan et al., 2008)). Acute inflammation self-resolves i.e. the inflammatory response comes to a stop. The resolution phase is characterised by the termination of PMN leukocyte emigration, fibrinolysis and the influx of monocytes which differentiate into macrophages (see for reviews: (Lawrence et al., 2002, Maskrey et al., 2011)). The resolution phase macrophages secrete chemo-attractants and pro-resolving mediators that stimulate phagocytosis of apoptotic cells (Stables et al., 2011). During this late phase, pro-inflammatory cytokine levels decline and the levels of anti-inflammatory cytokines such as tumour growth factor-beta (TGF β), IL-10 and IL-4 increase (see for review: (Lawrence et al., 2002)). The resolution of inflammation is important because failure of the inflammatory response to subside and self-resolve can lead to permanent tissue remodelling and chronic inflammatory diseases such as cardiovascular disease (CVD)

(see for review: (Osiecki, 2004)), rheumatoid arthritis (RA) (see for review: (Chimenti et al., 2015)) and Alzheimer's disease (AD) (see for review: (Rubio-Perez and Morillas-Ruiz, 2012)).

1.1.2 Chronic inflammation

Chronic inflammation is a result of continuous drive of the adaptive immune system. Ineffectual efficiency in the dampening of pro-inflammatory signals sustains and perpetuates inflammation. This leads to failed tissue healing (see for reviews: (Lawrence et al., 2002, Maskrey et al., 2011)). Chronic inflammatory diseases remain a human, economic and social burden on a global scale. Examples of chronic conditions include arthritis. According to Arthritis Research UK, 400,000 people in the UK suffered from arthritis with 2.46 million sufferers of hip osteoarthritis in England (Arthritis Research UK, 2014). In England, the prevalence of coronary heart disease (CHD) remained at 3% and hospital admissions increased by over 46,000 between 2010 and 2014 (Bhatnagar et al., 2016). In 2012, CVD was the leading cause of death in women. England spent approximately £6.8 billion between 2012 and 2013 (Bhatnagar et al., 2015) highlighting the need to control these conditions. Both RA and CHD reflect a chronic inflammatory condition, through activation of a circuit of signalling molecules, which, via positive feedback mechanisms, perpetuate disease (see for reviews: (Chimenti et al., 2015, Kraakman et al., 2016)).

It is therefore evident that there exists a necessity to explore the mechanism(s) by which the inflammatory response becomes protracted and an understanding of how the resolution phase is obscured; leading to chronic disease. This will unravel novel

therapeutic targets to instigate the resolution phase by understanding the transition from acute to chronic inflammation.

1.2 Macrophages in inflammation

The immune cell of interest in the current study was macrophages. This project was carried out using the macrophage J774.2 cell line. These are semi-adherent macrophages obtained from BALB/C mouse which partially adhere to the surface of the flask but can also be grown in suspension. Monocytes are derived from bone marrow progenitor cells and move from the blood into specific tissues. Here, they differentiate into macrophages and survey their immediate surroundings. Macrophages are imperative in physiology and pathophysiology as they contribute to a multitude of bodily changes. These cells form part of the innate immune system and are present in essentially all tissues; serving a diverse range of functions (see for review: (Chen and Zhang, 2017)). One such function which highlights the relevance of macrophage is wound healing. Wound healing is comprised of 4 stages: haemostasis (blood clot), inflammation (activation of the immune cells), proliferation (proliferation of keratinocytes and fibroblasts) and remodelling (macrophage release enzymes which remodel the extracellular matrix). Removal of macrophages during the mid-healing stage impairs the process whilst inducing haemorrhages. This highlights the importance of macrophages here (see for review: (Hesketh et al., 2017)).

A well-established crucial role of macrophages is phagocytosis and clearance of apoptotic granulocytes and effete cells (see for review: (Oishi and Manabe, 2016)). It has been suggested that dysfunctional macrophage phagocytic activity leads to impaired resolution (see 1.1.1); also highlighting its importance in aging and disease

(see for review: (Oishi and Manabe, 2016)). Despite being present in the resolution phase of inflammation, macrophages have typically been observed as the pro-inflammatory immune cell that produce pro-inflammatory cytokines and further activate other immune cells. In several chronic conditions, activated macrophages have been reported. Recent focus has been on macrophage phenotype present during the pro-inflammatory and resolution phase of inflammation.

1.2.1 Macrophage polarisation

Macrophage polarisation is a relatively new concept that has emerged from identifying plasticity in the macrophage phenotypes during the inflammatory response. The classically activated macrophages (CAM) are denoted M1 and activate the Th1 response, while the alternatively activated macrophages (AAM) are denoted M2 and activate the Th2 response (see for review: (Lawrence and Natoli, 2011, Sica and Mantovani, 2012, Tugal et al., 2013, Oishi and Manabe, 2016, Chen and Zhang, 2017)) (fig 1.2.1.1). The M1 subtype can be activated by pro-inflammatory stimuli such as TNF α and interferon gamma (IFN γ). Whereas it was believed that IL-4 and IL-13 were inhibitors of macrophage activation, it is now widely accepted that these anti-inflammatory cytokines activate a distinct subtype of macrophages (M2) (see for review: (Lawrence and Natoli, 2011, Hoeksema et al., 2012, Tugal et al., 2013, Liu et al., 2014, Chen and Zhang, 2017)) (fig 1.2.1.1).

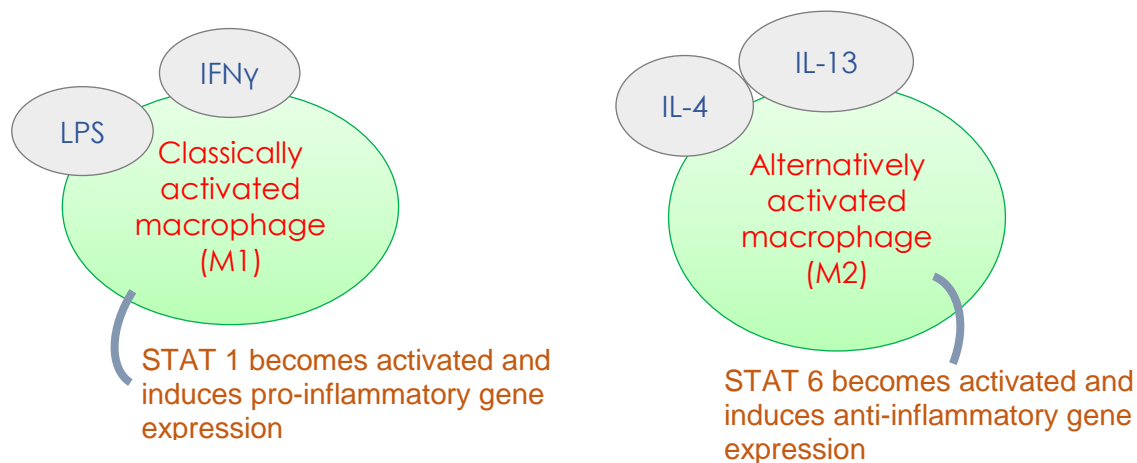


Figure 1.2.1.1: Diagram portraying the macrophage polarisation paradigm whereby macrophages take an anti-inflammatory or pro-inflammatory phenotype depending on the stimulus.

Interferon Gamma (IFN γ) and lipopolysaccharide (LPS) bind to their receptors, interferon gamma receptor and toll like receptor 4 respectively. This polarises the macrophages to classically activated M1 macrophages. This requires activation of STAT1. Alternatively activated M2 macrophage are obtained following IL-4 and IL-13 via STAT6 activation.

Both macrophage phenotypes express differential markers that facilitate the identification of the macrophage present (see for review: (Liu et al., 2014)); Following both *in vivo* and *in vitro* experiments, it has been suggested that the macrophage phenotype is dictated by the dominating inflammatory microenvironment (see for reviews: (Italiani and Boraschi, 2014, Van Overmeire et al., 2014)) which includes the cytokine profile (see for review: (Italiani and Boraschi, 2014)), lipid mediators (see for review: (Lawrence and Natoli, 2011)) and the other immune cells present (see for review: (Lawrence and Natoli, 2011, Italiani and Boraschi, 2014, Van Overmeire et al., 2014)). This highlights the role of the stimuli on the macrophage phenotypic nature.

Although cAMP was shown to be a critical determinant for the conversion of macrophages from a pro-inflammatory to an anti-inflammatory phenotype (Ghosh et al., 2016), this molecule was shown to be downstream to the initiation of resolution

(Bystrom et al., 2008). Bystrom et al. (2008) used an *in vivo* model for acute inflammation and showed that the macrophages found during the resolution phase are the same as the cells that had migrated into the inflamed site during the pro-inflammatory phase (~24 hours from onset of inflammation). This was determined using the phagocyte-specific dye PKH26-PCL^{red} and PKH26-PCL^{green}, as a tool to identify macrophage trafficking. There appears to be a novel induction pathway for both macrophage phenotypes through the activation of specific transcription factors such as signal transducer and activator of transcription (STAT) (see for review: (Chen and Zhang, 2017)) (fig 1.2.1.1). Despite this M1 M2 paradigm, depending on the site of inflammation, a mixed population of macrophages was reported. This macrophage heterogeneity showed embryonic and haematopoietic macrophages each with different receptors (see for review: (Gordon and Pluddemann, 2017)). The adaptability and plasticity of macrophages came from their receptor repertoire and versatile responsiveness to heterogenic environments (see for review: (Geissmann et al., 2010)). Use of antibodies for these receptors have strengthened the notion for macrophage heterogeneity (see for review: (Gordon and Taylor, 2005)).

1.2.2 Signalling pathways involved in macrophage polarisation

Macrophages are polarised to the pro-inflammatory M1 phenotype when IFN γ binds to its receptor, which activate the Janus kinase (JAK). JAK dimerise and cross phosphorylate each other at the tyrosine residue which phosphorylates the tyrosine residue of the receptor tail. The SH2 domain of inactivated STAT1, a pro-inflammatory transcription factor, recognises the phosphotyrosine sequence on the activated IFN γ receptor and binds to tyrosine phosphate receptors. The carboxy terminus of STAT1

becomes phosphorylated by JAK and dimerises; allowing translocation into the nucleus where it can drive the expression of pro-inflammatory genes.

To achieve the anti-inflammatory M2 phenotype, IL-4 (or IL-13) binds to its receptor. This activates JAK to phosphorylate and activate the STAT6 transcription factor which drives anti-inflammatory gene expression such as mannose receptor (see for reviews: (Lawrence and Natoli, 2011, Hoeksema et al., 2012, Tugal et al., 2013, Liu et al., 2014, Chen and Zhang, 2017). For robust polarisation to M2 macrophages by IL-4, cyclic adenosine monophosphate (cAMP) is critical, as it has been shown to increase expression of arginase (Arg-1) in microglial cells (Ghosh et al., 2016), and production of anti-inflammatory cytokines (Bystrom et al., 2008).

1.2.3 IL-4 signalling

IL-4 was initially regarded a stimulator of B cells and a survival factor for lymphocytes by protecting them from apoptosis (Luzina et al., 2012). Th2 cells, the analogy for M2 macrophages, produce IL-4, however, IL-4R was found on various cell types in addition to haematopoietic cells (Nelms et al., 1999). The tight control of IL-4 induced tissue repair was evaluated in bone marrow derived macrophages (BMDM), where apoptotic neutrophils were required for IL-4 mediated repair. Macrophages can detect these apoptotic cells by identifying phosphatidylserine (Bosurgi et al., 2017) which signals them to engulf the cells. This highlights the importance of IL-4 during the resolution of inflammation.

IL-4 is one of the key drivers of macrophage polarisation. IL-4 is well known for naïve cell differentiation where IL-4 drives Th2 differentiation and Th1 inhibition during

parasitic infection. These effects are primarily mediated via STAT6 transcription factor activation (Levings and Schrader, 1999). This cytokine regulates a profusion of biological functions e.g. proliferation and apoptosis. Thus, the pleiotropic downstream effects of IL-4 highlight its importance in immune responses. IL-4 has been reported to be protective in collagen induced RA murine models, which is an example of chronic inflammation (Morita et al., 2001), by dampening Th1 mediated responses. It mediates its effects via IL-4R which is expressed on a variety of haematopoietic and non-hematopoietic cells (see for review: (Zamorano J, 2003)).

The type 1 receptor consists of IL-4R with an α and γ chain. The IL-4R α chain does not possess enzymatic activities until IL-4 activation. The γ chain associates with IL-4R α following binding of the ligand. The heterodimerisation of the subunits initiate a signalling machinery via activation of kinases that associate with this complex.

It is lethal to block the pro-inflammatory STATs involved in M1 polarisation because blocking the production of pro-inflammatory genes would impair the ability to combat infections (see for review (Mitchell and John, 2005)). Furthermore, activating the M2 phenotype consistently will promote tumour formation similar to Th2 cells because of the tumorigenic properties of the M2 subtype (see for review: (Chen and Zhang, 2017)). Therefore, fully understanding how the transition from the pro-inflammatory macrophage to the resolution macrophage is inhibited in chronic inflammation would provide novel targets in the treatment of chronic diseases. A small but highly significant part of this is cyclooxygenase (COX)-2 and the prostaglandins (PG) involved in inflammation.

1.3 Production of prostaglandins (PG)

In the early 1990s, lipopolysaccharide (LPS)-treated human monocytes *in vitro* (Fu et al., 1990) and mouse peritoneal macrophages *in vivo* (Masferrer et al., 1990) were shown to up-regulate PG synthesis in inflammation. Lipid compounds called prostanoids are derived from the poly-unsaturated fatty acid, arachidonic acid (AA). AA is enzymatically obtained from the phospholipid through cleavage by phospholipase A (PLA₂). Prostanoids consist of PG, prostacyclin (PGI₂) and thromboxane (TXA₂). AA is metabolised by COX enzymes, also known as prostaglandin-endoperoxide synthase (PTGS). COX is an integral membrane glycoprotein that is abundant in the nuclear envelope and endoplasmic reticulum. COX has two active sites: the COX active site and the peroxidase active site (see for reviews: (Bjorkman, 1998, Simmons et al., 2004)). COX enzymes must be in their oxidised state to be active. Tyrosine-385 in the COX active site of the enzyme is oxidised by a tyrosyl radical (Hanel and Lands, 1982). This is generated at the peroxidase active site through the reduction of a lipid hydroperoxide (LHP) substrate. Constant supply of LHP therefore sustains COX in its oxidised state. This generates hydroperoxy endoperoxide prostaglandin G₂ (PGG₂) which is reduced to PGH₂ via the peroxidase reaction. PGH₂ is the precursor for the synthesis of PGE₂, PGI₂, PGD₂, PGF_{2α} and TXA₂ which are ubiquitously produced and biologically active (Bjorkman, 1998).

Opposing PG functions and varied temporal release throughout the inflammatory process has been recorded. Each PG are known to play a significant role in governing the inflammatory response (see for review: (Willoughby et al., 2000)). Augmented levels of PGE₂ and PGI₂ in inflamed tissues contribute to the cardinal signs

of inflammation (see for review: (Ricciotti and FitzGerald, 2011)). PGE₂ has been shown to have a key role in exudate formation in acute inflammation (Katori et al., 1998). PGD₂ is predominant in the brain and is metabolised into the J series cyclopentanone PG (cyPG), 15-Deoxy-Delta-12,14-prostaglandin J₂ 15d-PGJ₂, via a dehydration step (see for review: (Scher and Pillinger, 2005)). Thus, it is clear that there is temporal emphasis on PG synthesis and this is key to impacting the course of inflammation.

1.4 The discovery of the COX-2 protein and its role in inflammation

Research into the COX enzymes peaked in the 1990s with the discovery of an inducible COX isoform. Initially, a 2.7 kb COX protein was identified and regarded as the only COX enzyme present. Habenicht et al. (1985) induced this enzyme in mouse Swiss 3T3 fibroblasts with platelet-derived-growth-factor (PDGF) and observed 2 PG peaks; one at 10 minutes and another at 2-4 hours. Addition of the translational inhibitor, cycloheximide, blocked only the late peak, suggesting a pool of induced COX protein. Fu et al. (1990) revealed that dexamethasone (DEX) was more able to inhibit LPS-induced COX activity compared to unstimulated COX in monocytes, reinforcing the potential for a pool of COX with differential properties. DEX is a glucocorticoid (GC) that down-regulates inflammation. LPS is found on the outer membrane of gram negative bacteria and elicits an immune response. Xie et al. (1991) provided compelling evidence for an inducible ~5.0 kb COX enzyme, whose features were distinct from that of the 2.7 kb COX. This was represented by biphasic elevation of ~5.0 kb COX in fibroblasts. Interestingly, Xie et al. (1991) showed that in the presence of translational inhibitors, inducible COX mRNA increased, alluding that this ~5.0 kb COX is synthesised de novo.

Two COX isoforms have been identified: COX-1 which is expressed constitutively and COX-2 which is inducible (Xie et al., 1991, Katori et al., 1998). Constitutive COX-1 has primary roles in physiological function such as gastric cyto-protection by producing PGE₂ and PGI₂ which mediate vasodilation and reduced gastric acid secretion (see for reviews: (Bjorkman, 1998, Simmons et al., 2004)). COX-2 enzymes are induced by pro-inflammatory cytokines and are highly expressed and active during the inflammatory response (Lawrence et al., 2002). Ongoing research has provided mounting evidence for the involvement of COX-2 in pyrexia, pain, CVD, cancer and AD (see for review: (Simmons et al., 2004)). This knowledge has been a key clinical advancement, as it provided a new drug target for several inflammatory conditions.

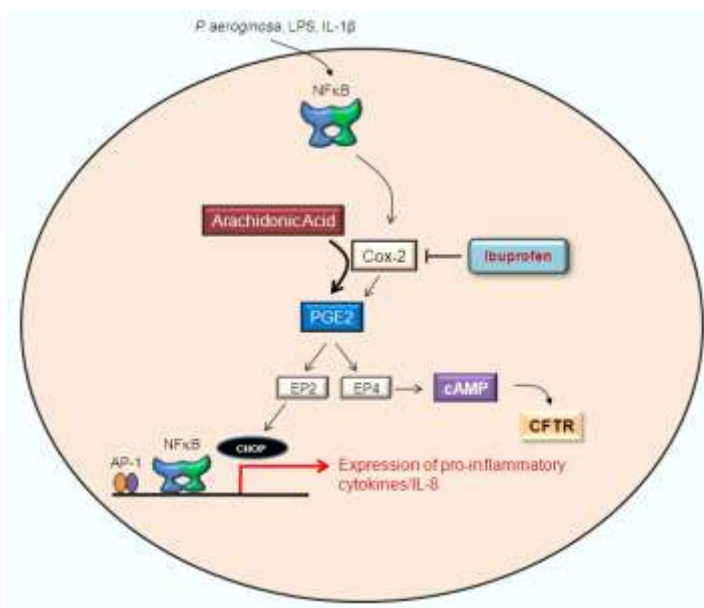


Figure 1.4.1: Diagram showing the induction of COX-2 when LPS binds to its receptor.

Following the binding of a pro-inflammatory molecule such as LPS to its receptor, pro-inflammatory transcription factors such as NFκB become activated. This induced COX-2 and stimulates the release of arachidonic acid from the phospholipid bilayer. COX-2 produce prostaglandin products which have various effects depending on the receptor it binds to. Image taken from (Bodas and Vij, 2010).

1.5.1 Biphasic expression of COX-2

In rat carrageenan induced pleurisy model for acute inflammation, during the early phase, high levels of PGE₂ were observed followed by a shift towards PGF_{2α} as the response waned (Capasso et al., 1975). This again, highlights the temporal factor of PG synthesis throughout the course of inflammation. Using the same model as Capasso et al. (1975), Gilroy et al. (1999) reported induction of COX-2 protein with maximal PGE₂ release at 2 hours, where PMN leukocytes are dominant. This was followed by marginal PGE₂ synthesis and a second peak in COX-2 protein expression at 48 hours; 350% greater than that observed at 2 hours. This biphasic paradigm of COX-2 induction was previously reported in chicken embryo fibroblasts (CEF) at 1 hour and again to a higher level at 4 hours (Xie et al., 1991). This again highlights the temporal regulation on the expression of COX-2. Gilroy et al. (1999) showed that PGD₂ and 15d-PGJ₂ levels peaked at 2 hours followed by a decline during the course of inflammation and a subsequent incline at 48 hours. It is yet to be clarified, whether endogenous concentrations of 15d-PGJ₂, being a natural ligand for peroxisome proliferator activated receptor gamma (PPAR γ), initiates an anti-inflammatory route for COX-2 function. Bystrom et al. (2008) reported COX-2 expression on what was regarded as resolution macrophages, despite its expression being observed on pro-inflammatory macrophages (see for review: (Sica and Mantovani, 2012)).

1.5.2 COX-2 inhibition amplifies inflammation

COX-2 inhibitors were shown to exacerbate disease by blocking PG (see for review: (Yedgar et al., 2007)). Attenuated inflammation was observed at 2 hours following the addition of NS-398 and indomethacin; a selective COX-2 inhibitor and a dual COX-1/2 inhibitor respectively, (Gilroy et al., 1999). Exacerbated inflammation

was detected at 48 hours with reduced 15d-PGJ₂ levels, following treatment with NS-398. The authors were unable to detect COX-1 and suggested late induced COX-2 to govern the resolution phase. These findings agree with Chan and Moore (2010) who reported COX-2 mRNA to be elevated during the resolution phase in murine collagen induced arthritis, another inflammatory model. NS-398, given in early stages, attenuated arthritic symptoms while inflammation was perpetuated when given in later stages (Chan and Moore, 2010). These key findings emphasise the temporal importance of COX-2 expression and the time of NSAID administration.

Pulmonary expression of COX is elevated in ovalbumin induced allergic inflammation and it was commonly conceived that COX products had pernicious effects on the lung. Despite this, PGE₂ was found to subdue immunoglobulin (Ig)E production and block allergen induced asthmatic responses (Gavett et al., 1999). Lungs from COX-2 deficient mice exhibited inflammation of greater intensity compared to the wild-type mice. These mice also displayed impaired alveolar septae. When measuring the degree of lung inflammation, it was evident that COX-2 deficient mice had a significantly greater degree of lung inflammation (Gavett et al., 1999). This was in many ways similar to work carried out by Wallace et al. (2000). Wallace et al. (2000) showed COX-2 derived PG to contribute to the gastric defense system. It was made clear that non-steroidal anti-inflammatory drug (NSAID) blockage of COX-2 as well as COX-1 contributed to mucosal damage. Collectively, these studies highlight the role of COX-2 and PGE₂ in the resolution of the acute inflammatory response.

Whereas in COX-2 deficient mice, no clinical manifestations were reported and the colon mucosal integrity was preserved (Morteau et al., 2000), the addition of

dextran sodium sulfate (DSS) induced colitis. COX-2 deficiency showed reduced PGE₂, as expected. Morteau et al. (2000) found lack of COX-2 to accelerate severe colitis, as shown by aggressive injury; whilst also increasing death. This may have been driven by COX-2 deficient IL-1 β induction, which is produced as part of the innate immune response (Morteau et al., 2000). Morteau et al. (2000) predicted that COX-2 derived PGE₂ is therefore critical in aggressive colitis, as it assists with the healing process and encourages mucosal protection.

Skeletal muscle cells that were exposed to palmitate and NS-398 exhibited increased levels of IL-6 and TNF α mRNA with reduced PGE₂, compared to cells treated with palmitate alone (Coll et al., 2010). Furthermore, when PGE₂ was co-treated with NS-398 and palmitate, induction of nuclear factor kappa B (NF κ B), a transcription factor involved in pro-inflammatory pathways, was attenuated. This was not observed in the absence of PGE₂, thus suggesting PGE₂ to mediate this anti-inflammatory effect. PGE₂ was also able to reduce TNF α when these cells were treated with NS-398 and palmitate. Coll et al. (2010) proposed that increased AA, due to blockage of COX, was available to be metabolised into 12-hydroxyeicosatetraenoic acid (12-HEPE) which evokes pro-inflammatory effects (Coll et al., 2010).

The anti-inflammatory properties of the COX-2 derived PGE₂ suggest the 'late' COX-2 may be different to the 'early' COX-2. The effects seen above may be due to inhibition of COX-2 with different biochemical properties.

1.6 Sensitivity of COX-2 for inhibition

1.6.1 Inhibition of COX-2 by acetaminophen

Simmons et al. (1999) gave evidence for acetaminophen (paracetamol) to inhibit diclofenac induced COX-2 activity in a dose dependent manner; however, this effect was not observed in LPS-induced COX-2. Furthermore, LPS induced COX-2 was more sensitive to NSAIDs compared to the diclofenac induced COX-2 (Simmons et al., 1999). Ten years later, Ayoub et al. (2009) observed comparable findings, suggesting two induced COX-2 proteins which possess different selectivity for inhibition.

Acetaminophen is widely used as a first line of treatment for antipyretic and analgesic therapy and can be obtained over the counter (see for reviews: (Botting, 2000, Sharma, 2013)). To date, the mechanism by which acetaminophen produces these effects remains ambiguous, however studies have reflected acetaminophen as a weak inhibitor of COX enzymes (Mitchell et al., 1993). Suggestions have been made associating limited anti-inflammatory properties with decreased COX inhibition in the periphery because of a high LHP tone; which is the intermediates of lipid peroxidative reactions. This differs to the CNS where there is a low LHP tone and COX activity can be inhibited by acetaminophen, thus acetaminophen has antipyretic and analgesic properties. As mentioned previously, COX activity requires the enzyme to be in an oxidised state and acetaminophen is a reducing agent that reduces COX into an inactive state, thereby reducing PG synthesis (Ouellet and Percival, 2001). This paradigm may explain the increased potency of acetaminophen to inhibit COX enzyme activity in dog and rabbit brain compared to dog spleen (Flower and Vane, 1972). In contrast, Ayoub et al. (2011) showed diclofenac induced COX-2, but not LPS induced

COX-2, in J774.2 macrophages to be inhibited by acetaminophen regardless of the LHP tone (Ayoub et al., 2011). A high LHP tone induced by J774.2 macrophages treated with diclofenac and a LHP donor (T-butyl hydroperoxide), did not antagonise the inhibition of diclofenac induced COX-2 by acetaminophen. On the other hand, acetaminophen did not inhibit LPS-induced COX-2 where the LHP remained relatively low (Ayoub et al., 2011). NSAIDs, however, behaved as potent inhibitors of LPS induced COX-2 but not diclofenac induced COX-2 (Simmons et al., 1999). This suggests that diclofenac induced COX-2 may be biochemically different hence the differential selectivity for inhibition by acetaminophen.

1.6.2 Inhibition of COX-2 by Indomethacin

COX-2 is the target for NSAIDs and has been accepted in the medical community for the treatment of chronic inflammatory diseases (Maskrey et al., 2011). The clinical efficacy of NSAID and their inhibitory properties on prostanoids stress the precedence of these mediators in pain, fever and inflammation (see for review: (Ricciotti and FitzGerald, 2011)). This class of drugs produce both therapeutic (Katori et al., 1998, Kotilinek et al., 2008) and toxic activities (see for review: (Hoppmann et al., 1991)) by inhibiting PG synthesis.

NSAIDs bind to the COX site, with no effect on the peroxidase site, to shut down prostanoid generation. This can only occur at one of the monomers that form the COX dimer. Aspirin is a non-selective NSAID that diffuse into the COX active site and acetylates serine-530 in COX-1 irreversibly. Aspirin inhibition of COX-2 is somewhat less efficient due to the larger catalytic pocket (see for reviews: (Bjorkman, 1998, Vane and Botting, 1998, Simmons et al., 2004)). Development of NSAIDs that share the

therapeutic aspect of aspirin was targeted by the pharmaceutical industry. To this end, indomethacin was produced to serve this purpose. Inhibition of COX-1 by NSAIDs interfere with the cellular function and cause adverse side effects such as gastrointestinal toxicity (see for review: (Kawai, 1998, Ricciotti and FitzGerald, 2011)). Selective COX-2 inhibitors such as celecoxib, have been developed to achieve antipyretic, analgesic and anti-inflammatory effects, without the side effects seen with non-selective COX inhibition (Warner et al., 1999).

Wallace et al. (2000) confirmed that celecoxib inhibited COX-2 without impacting COX-1, while SC-560 inhibited COX-1 without impacting COX-2. Of interest, indomethacin, the drug used by Gilroy et al. (1999), was reported to inhibit COX-1 and COX-2 to similar amplitudes. Thus, indomethacin behaves as a pan COX inhibitor. Derivatives of indomethacin were found to bind to COX (Rommel et al., 2004). Although IL-1 β and COX-2 were induced in pentylenetetrazole (PTZ) evoked seizures in zebra fish, exposure to indomethacin before PTZ treatment forced a down-regulation in both IL-1 β and COX-2. Administration of indomethacin also reduced the behavioural signs associated with seizures (Barbalho et al., 2016). Thus, indomethacin was used in this project to inhibit COX-2.

1.6.3 Inhibition of COX-2 by dexamethasone

DEX is a GC that has been widely recognised as an effective treatment method in the prevention of rejection in transplants, inflammatory and autoimmune disease. GC were not produced with the intention of relieving pain; rather they served to repress inflammation (see for review: (Punchard et al., 2004)). The clinical efficacy of DEX becomes paradoxical as long-term treatment leads to diabetes, osteoporosis and

some other inflammatory conditions (see for review: (Coutinho and Chapman, 2011)). Furthermore, a drawback of cortisol and synthetic GC agents like DEX was that they produced therapeutic effects at supra-physiological concentrations therefore, adversities were ineluctable (see for review: (Punchard et al., 2004)).

GC exerts its effects by binding to glucocorticoid receptor (GR) which behave as a regulator of gene expression. GR homodimerization allow it to bind to GC-response elements (GRE)s. GR activation was reported to dampen PPAR γ expression (Inoue et al., 2000), a receptor which was shown as a requirement for COX-2 induction by diclofenac (Ayoub et al., 2009). It is believed that GC mediated immunosuppressive effects are attributed to repressed transcription of immunomodulatory factors such as NF κ B. GC reduce vasodilation and oppose increased permeability which is seen during inflammation. Furthermore, GC was reported to reduce leukocyte emigration (see for review: (Coutinho and Chapman, 2011)). DEX exerts some of its anti-inflammatory effects, such as IL-10 production, via phosphorylation of ERK (Xia et al., 2005). GC induced IL-10 has been commonly noted in monocytes and macrophages (see for review: (Coutinho and Chapman, 2011)). Furthermore, it remains enigmatic whether IL-10 is produced because of GC induced differentiation or whether this IL-10 amplifies some of GC effects (see for review: (Coutinho and Chapman, 2011)).

In LPS treated keratocytes, the levels of TNF α , IL-6 and IL-1 β was elevated. Following a 48-hour DEX treatment, levels of these pro-inflammatory cytokines dropped significantly (Yan et al., 2017). Similarly, whereas LPS stimulated TNF α in PMN leukocytes from new borns, this was inhibited 55% by DEX. IL-1 β induced by LPS was inhibited 70% by DEX. This concentration was unable to impact

chemokinesis and chemotaxis; thus, positing its anti-inflammatory effects are mediated by impacting the cytokine release. Therefore, DEX may exert its anti-inflammatory effects on COX-2 in a similar manner.

On top of the anti-inflammatory role of DEX, this GC has also been associated with COX-2. Masferrer et al. (1992) gave evidence for DEX to regulate only inducible COX in sham-adrenalectomised kidneys. This steroid had no effect on the constitutive COX. LPS is an entrenched inducer of COX-2. Typically, DEX has been regarded to work antagonistically to LPS induced effects (Fu et al., 1990, Barrios-Rodiles and Chadee, 1998, Inoue et al., 2000, Abraham et al., 2006, Yano et al., 2007). As such, DEX blocks COX-2 induction (Barrios-Rodiles and Chadee, 1998). As we characterize the LPS induced 'early COX-2' and the 'late COX-2' as different, the effect of DEX on these two inductions of COX-2 is key.

1.7 Transcription factors involved in COX-2 induction

The transcription factors NF κ B and PPAR γ are believed to be involved in COX-2 protein induction, depending on the stimuli (Ayoub et al., 2009). NF κ B was shown to shut down PPAR γ (Chistyakov et al., 2015) suggesting a paradoxical relationship as both transcription factors are associated with the induction of COX-2.

1.7.1 NF κ B in inflammation

NF κ B has been suggested to be the "master" regulator of the inflammatory response (see for review: (Lin et al., 2017)). This is pertinent to the induction of COX-2 by LPS and also a member of the group of transcription factors involved in polarisation of macrophages towards the M1 phenotypic state. The inhibitor of NF κ B

(I κ B) kinase (IKK) phosphorylation-directed degradation of I κ B activates NF κ B which translocates to the nucleus and induces pro-inflammatory gene expression. Such target genes include TNF α and IL-1 which contribute to tissue damage (see for review: (Luo and Zhang, 2017)). Through a positive feedback program, TNF α increases NF κ B by binding to the TNF α receptor (see for review: (Luo and Zhang, 2017)).

1.7.2 PPAR γ transcription factor

PPAR γ is one of three types of steroid nuclear receptors with roles in glucose metabolism and inflammation. Following activation, PPAR- γ forms heterodimers with retinoid-X receptor to drive gene transcription and has pro-apoptotic properties (Wick et al., 2002). A class of drugs, called thiazolidines, are used in the treatment of diabetes. These drugs primarily increase insulin secretion from the pancreas by targeting PPAR γ .

Low levels of PPAR- γ have been reported in unstimulated macrophages followed by a subsequent increase in activated peritoneal macrophages (Rossi et al., 2000). In AD brains, increased hippocampal PPAR γ during inflammation was suggested to be a compensatory mechanism for a decrease in anti-inflammatory mediators (Wang et al., 2014). This suggests a potentially anti-inflammatory role for this receptor in inflammation.

Microarray analysis showed IL-4 induced STAT6 to be required for PPAR γ target gene expression (Szanto et al., 2010). Studies have given evidence for IL-4 to induce STAT6 and bind to the promoter region of PPAR γ target genes e.g. fatty acid binding protein (FABP4) (see for reviews: (Szanto et al., 2010, Sica and Mantovani,

2012, Tugal et al., 2013)). Furthermore, IL-4 induced STAT6 activates kruppel-like factor 4 (KLF4), another transcription factor. This was found to induce PPAR γ (Liao et al., 2011) (see for reviews: (Sica and Mantovani, 2012, Tugal et al., 2013)). Thus, whereas LPS reduced KLF4, IL-4 significantly increased this (Liao et al., 2011); reiterating a signalling pathway between IL-4 and PPAR γ . STAT6 deficiency stops the ability to inhibit pro-inflammatory cytokines such as TNF α (Levings and Schrader, 1999) and showed the inability to express M2 markers such as Ym1 (Szanto et al., 2010) (fig 1.2.2.1). Similarly, KLF4 deficient macrophages significantly reduce PPAR γ levels (Liao et al., 2011). This emphasises the anti-inflammatory role of both IL-4 and PPAR γ in inflammatory cell phenotype.

IL-4 binding to its receptor has been shown to increase 12/15-lipoxygenase activity which produces the endogenous ligand for PPAR- γ , 15d-PGJ₂ (Szanto et al., 2010). Interestingly, repression of LPS-induced NF κ B transcription was noted in the presence of PPAR- γ and the COX-2 product, 15d-PGJ₂, which activates PPAR- γ (Ricote et al., 1998). This implies a signalling axis between the anti-inflammatory cytokine and nuclear receptor that shuts down the pro-inflammatory transcription factor NF κ B.

1.7.3 Relevance of PPAR γ and NF κ B in COX-2 induction

Diclofenac is an NSAID and has previously been observed as an inducer of COX-2, in macrophage J774.2 cells (Simmons et al., 1999, Ayoub et al., 2009). Treatment of this cell line with diclofenac stimulated the cells to produce the anti-inflammatory cytokines: TGF β and IL-10 with low levels of pro-inflammatory cytokines: IL-6 and TNF α . Whereas LPS induced COX-2 as early as 6 hours, diclofenac could

only induce COX-2 at 48 hours and this COX-2 was termed the 'late COX-2' (Ayoub et al., 2009). In the diclofenac induction of COX-2 pathway, NF κ B inhibition had no notable effect on COX-2 expression but blocked LPS induced COX-2 (Ayoub et al., 2009). The PPAR γ antagonist, Bisphenol A diglycidyl ether (BADGE), reduced diclofenac, but not LPS-induced COX-2 expression. These results suggest PPAR γ is required for diclofenac-induced COX-2 protein induction while NF κ B is key in the LPS pathway leading to the expression of COX-2 (Ayoub et al., 2009). This study along with research conducted by Gilroy et al. (1999) and Lawrence et al. (2001) provide compelling evidence towards a pool of 'late COX-2' distinct from the 'early COX-2'.

1.8 Mediators involved in the induction of COX-2

1.8.1 Mitogen-activated protein kinases (MAPK)

Some studies reflect a relation between PPAR γ and a protein kinase. Mitogen activated protein kinases (MAPK) are key players in inflammation and have been identified as targets in chronic inflammation (Campbell et al., 2004) (see for reviews: (Cuenda and Rousseau, 2007, Huang et al., 2009, Cuadrado and Nebreda, 2010, Fisk et al., 2014)). The MAPK relay extracellular signals into intracellular responses via phosphorylation and engender a plethora of effects. This sophisticated relay mechanism allows for appropriate responses to be orchestrated. Phosphorylation is the transfer of a phosphate group from a phosphate donating molecule such as ATP to the threonine (thr), tyrosine (tyr) and/or serine (ser) of a substrate. Conventional MAPK include, ERK1, ERK2, JNK1, JNK2, JNK3, p38 which exists in 4 isoforms: α , β , γ and δ . P38 α and β are expressed ubiquitously in tissues however p38 γ and δ are not. These MAPK can be activated by various stimuli including environmental stress,

growth factors, hypoxia, ischaemia and cytokines (see for review: (Zarubin and Han, 2005)(Huang et al., 2009)).

There are 3 evolutionarily conserved kinases which act in a sequential manner. MAPK, MAPK kinase (MAP2K) and MAPKK kinase (MAP3K). Following activation by a stimulus, example $\text{TNF}\alpha$, MAP3K becomes activated. This phosphorylates ser/thr region and activates the MAP2K: either MKK3 or MKK6. MAP2K activate p38 via phosphorylation. This phosphorylation occurs on the activation loop at the Thr-Gly-Try motif. Activated p38 phosphorylates and activates MAPKAPK-2 (MK2), MAPKAPK-3 (MK3) as well as various other transcription factors. These are collectively termed MAPK-activated protein kinase (MAPKAPK).

1.8.1.1 p38 MAPK

This research shows particular interest in p38 MAPK due to its relevance in inflammatory signalling pathways and regulation of PPAR γ . p38 has historically been associated with the production of pro-inflammatory cytokines by regulating their expression via transcription factor regulation such as NF κ B or by modulating the mRNA stability and translation (see for review: (Cuenda and Rousseau, 2007, Coulthard et al., 2009, Cuadrado and Nebreda, 2010)). p38 α activation is stimuli and cell type specific. p38 α has been shown to possess pro-inflammatory properties as shown in RA patients (Korb et al., 2006) and can be phosphorylated downstream to LPS binding its receptor. Although LPS activates p38, p38 does not stabilise all LPS target genes. Despite genes being destabilised by a p38 inhibitor, they were not upregulated by LPS (Briata et al., 2005). This suggests both p38 and LPS do not share a mutualistic relationship i.e. they do not activate each other.

A large body of evidence pointed towards p38, specifically the α isoform, to be pro-inflammatory. This led p38 to be considered as a promising drug target in chronic inflammation. Korb et al. (2006) used immunoprecipitation to show the α and γ isoform to be activated and expressed in RA synovial fluid but not in the control. The compound SB203580 acts as a specific competitive ATP binding inhibitor for p38 α and β (Davies et al., 2000). This compound has been used in animal models for RA, AD, inflammatory bowel disease among others, due to its ability to reduce pro-inflammatory cytokine expression (see for review: (Kumar et al., 2003, Fisk et al., 2014)). Nevertheless, safety risks reflected p38 MAPK as a poor therapeutic target (Denise Martin et al., 2012).

Despite the general view that p38 is pro-inflammatory, a recent publication has provided controversial data showing p38 inhibition to suppress typical anti-inflammatory macrophage markers: Arg-1, Ym-1 and Fizz-1 (Jimenez-Garcia et al., 2015). Additionally, inhibition of p38 inhibited phosphorylation of STAT6, a key transcription factor involved in AAM (see 1.2.1.1). Inhibition of p38 leading to inhibition of phospho-STAT6 expression suggests that STAT6 is activated downstream to p38 MAPK in an anti-inflammatory signalling cascade (Jimenez-Garcia et al., 2015). The data from this paper provided both *in vitro* and *in vivo* evidence for the involvement of p38 MAPK in polarisation towards the AAM. In conjunction with this, Kim et al. (2008) showed p38 α dependent expression of IL-10 amongst its tier of target genes; thus, accentuating a role for this kinase in anti-inflammatory pathways. This may explain poor therapeutic benefits inspected by Guma et al. (2012) when p38 α was blocked in macrophage dominating inflammatory disease.

p38 MAPK has been associated with PPAR γ making it highly relevant in this project. Schild et al. (2006) showed that p38 inhibition reduced PPAR γ activity in human primary trophoblasts whereas PPAR γ did not affect p38 MAPK expression. Moreover, p38 was required for PPAR γ stability as p38 inhibition reduced the expression of this transcription factor. Thus, Schild et al. (2006) gave evidence for p38 MAPK to regulate PPAR γ expression and activity. Correspondingly, both Yano et al. (2007) and Chistyakov et al. (2015) corroborated the regulatory control of p38 on PPAR γ because the introduction of SB203580 inhibited PPAR γ .

1.8.2 cAMP as a second messenger

Like p38 MAPK, interest in cAMP was shed due to its involvement in immune function (see for review: (Daniel et al., 1998)). Integrating extracellular and intracellular signals, cAMP allows cellular adaptation by amplifying the signal of the initial stimulus. cAMP is a potent regulator of adaptive and innate immune functions. It is key in pathogenic disease and so a promising therapeutic drug target (see review: (Raker et al., 2016)). Elevated levels of cAMP typically weaken pro-inflammatory responses and phagocytosis (see for review: (Serezani et al., 2008, Yan et al., 2016)). Concomitantly, cAMP encourages increase in anti-inflammatory markers and cytokines (Bystrom et al., 2008). In a self-contained system, cAMP increase phosphodiesterase (PDE) activity, which negatively regulate cAMP by degrading intracellular cAMP. Thus, PDE behave as an inflammatory response mediator (see for review: (Omori and Kotera, 2007)) and PDE inhibitors can curtail the inflammatory response. Development of PDE inhibitors, although an attractive approach, has demonstrated excessive side effects including abdominal pain and nausea (see for review: (Raker et al., 2016)).

PGE₂ is a ligand for G protein-coupled receptor (GPCR). Being a product of COX-2, it is believed to possess pro-inflammatory properties. Binding of PGE₂ to its 7 transmembrane-spanning GPCR leads to the exchange of a phosphate molecule from guanine triphosphate (GTP) to guanine diphosphate (GDP). The ligand binding forces a conformational change and the disassociation of G protein alpha subunit (G α s) from the β and γ subunit; which, activates adenylyl cyclase (AC). AC is responsible for the generation and modulation of cAMP by catalysing the conversion of adenosine triphosphate (ATP) to cAMP (see for review: (Serezani et al., 2008, Yan et al., 2016)). Activated cAMP can bind the regulatory subunit of protein kinase A (PKA). The catalytic subunit of PKA serves to phosphorylate serine and threonine sites of target cAMP response element binding proteins. H89 is an inhibitor of PKA which blocks LPS induced PGE₂ and blocks MAPK by inhibiting the CREB mediated mRNA (see for review: (Yan et al., 2016)). Thus, the significance of cAMP in both PGE₂ and p38 mediated effects, makes this molecule key in the project.

1.8.2.1 The role of cAMP in macrophage polarisation

cAMP is recognised to abate a multitude of inflammatory responses (see for reviews: (Serezani et al., 2008, Raker et al., 2016, Yan et al., 2016)). PDE4 inhibitors led to the inhibition of pro-inflammatory molecules such as TNF α and NF κ B (see for review: (Spadaccini et al., 2017)) via cAMP upregulation, suggesting cAMP to have some regulatory/stimulatory effects on cytokines. cAMP has been targeted as a mediator in macrophage polarisation as, addition of dibutyryl, the cAMP analog (db-cAMP), to pro-inflammatory macrophages showed an increase in IL-10 and reduced TNF α production. In the presence of rp-cAMP, the cAMP antagonist, TNF α production was increased (Bystrom et al., 2008). Thus, Bystrom et al. (2008) showed the

macrophage phenotype to be altered by variations in the intracellular cAMP levels by using agonists. The conversion from M1 to M2 may be mediated by PKA, as PKA specific cAMP analogs co-treated with IL-4, induced Arg-1 in M1 microglial cells (Ghosh et al., 2016).

It is evident that cAMP provides synergism with IL-4 in polarising macrophages from an M1 to an M2 phenotype. This gave impetus to further understand the relevance of cAMP in macrophage polarisation and the expression of COX-2 in the anti-inflammatory macrophages.

There is evidence suggesting that binding of PGE₂ to the EP₄ receptor exerts anti-inflammatory downstream effects (Sokolowska et al., 2015) (see for review: (Luo and Zhang, 2017)). Pre-treatment of cells with PGE₂ inhibits LPS induced NFκB1 p105 phosphorylation in mouse BMDM via EP₄ (Minami et al., 2008). Silencing the EP₄ associated proteins in RAW264.7 cells show impaired inhibition of LPS induced p105 phosphorylation. This suggests that targetting the PGE₂-EP₄ receptor axis, removes the EP₄ induced inhibition on LPS induced NFκB activation (Minami et al., 2008). EP₄ knockout mice showed significantly increased cellular infiltration following LPS challenge (Minami et al., 2008). This suggests PGE₂ acting via the EP₄ receptor to possess an endogenous anti-inflammatory function. This agrees with previous studies that have shown EP₄ to reduce TNFα production (Katsuyama et al., 1998). Low levels of Ono-AE2-227, the EP₄ antagonist, induces a right-hand shift in TNFα inhibition against the log concentration of PGE₂. This suggests EP₄ to be the dominant receptor that mediates the anti-inflammatory effect of PGE₂ (Ratcliffe et al., 2007). HEK cells that were treated with Ono-AE2-227, inhibited PGE₂ induced cAMP elevations

(Ratcliffe et al., 2007). Collectively, this shows a strong and potentially significant relation between COX-2 induced PGE₂, EP₄ and cAMP.

1.9 Hypothesis

Selective inhibition of the late induced COX-2 but not the early COX-2 by acetaminophen *in vitro*, suggests the two COX-2 proteins to possess different biochemical properties. Opposing responses from *in vivo* inhibitory experiments leads us to believe these COX-2 proteins are in fact different; with tight temporal regulation for expression *in vitro*. We believe that the induction pathway for COX-2 by IL-4 is different to the classical LPS induced COX-2 induction pathway. Despite p38 commonly being associated with COX-2 in pro-inflammatory pathways, we sought to find an anti-inflammatory role for p38 in IL-4 induced COX-2. Furthermore, interest in PPAR γ developed from its requirement for the induction of COX-2 by diclofenac. As both diclofenac and IL-4 induce COX-2 at a later time point, we hypothesise PPAR γ to also be a requirement for IL-4 induced COX-2 *in vitro*. We hypothesise the nature of the COX-2 to be dependent on the microenvironment and phenotype of the macrophages present. As such, we believe p38 and PPAR γ to also be required for the production of the anti-inflammatory cytokine, IL-10. The relevance of cAMP in macrophage polarisation has been well established. In this project, we predict cAMP to be relevant for the induction of COX-2 by IL-4 in macrophages that we assume to be anti-inflammatory.

1.10 Aims and objectives

The aim of this project was to investigate whether the anti-inflammatory cytokine, IL-4 could induce a catalytically active COX-2 protein and whether this was

dependent on both p38 MAPK and PPAR- γ . Understanding this will facilitate our understanding on how resolution fails in chronic inflammation. Furthermore, the dependency of p38 and PPAR γ to induce a catalytically active COX-2 with anti-inflammatory properties, will open new drug targets.

This study aimed to draw parallel between the induction of COX-2 and the polarisation state of the macrophage. Accordingly, this work will analyse the effect of COX inhibition by an NSAID and GC on the polarisation state; as defined by the cytokine profile of the macrophage. The role of cAMP in both the LPS induced pro-inflammatory pathway and the IL-4 induced anti-inflammatory pathway will be examined.

As we speculate that both the IL-4 and LPS induced COX-2 to be “different” due to the differed induction times, different induction pathways and different sensitivities for inhibition properties, we sought to scrutinise the induction pathway through various pharmacological inhibition & antagonism assays. To this end, we aimed to gain further clarification on the following:

- 1) Identify an endogenous inducer of a late COX-2 protein in macrophage J774.2 cells.
- 2) Analyse the sensitivity of inhibition for COX-2 that is induced by LPS, diclofenac and IL-4 in an attempt to identify the potentially different biochemical properties.
- 3) Determine the sequence of events that lead to the activation of COX-2 and the production of PGE₂ by IL-4.
- 4) Clarify the role of cAMP in COX-2 induction by IL-4.
- 5) Confirm the effect of both LPS and IL-4 on the cytokine profile of the macrophage as a measure of the phenotype.
- 6) Investigate whether addition of indomethacin or DEX has differential effects on the COX-2 induced by LPS and IL-4; whilst also correlating the phenotype of the macrophage with changes in the cytokine profile.

Chapter 2: Methods

2.1 Cell Culture

2.1.1 General maintenance of the macrophage J774.2 cells

All cells were cultured in the same way unless otherwise stated. Macrophage J774.2 were grown in Dulbecco's modified eagle medium (DMEM) (Lonza) that contained high glucose (4.5g/l), 10% fetal bovine serum (FBS) (Sigma), 5% antibiotics (penicillin and streptomycin) and antifungal (Amphotericin B) (Sigma). To dislodge cells from the surface of the flask, scrapers were used. The content was spun at 500g at 4°C for 5 minutes. The supernatant was decanted and the pellet re-suspended in a volume of DMEM. A percentage of this volume was distributed into new flasks for sub-culturing or experimental purposes. The flasks containing the cells were placed in incubators with the following conditions at all times: 5% CO₂, 5% humidity and 37°C.

2.1.2 Experimental cell stimulations

Depending on the nature of the experiment and the required total cell number, either a T25 flask or plates with 6 or 24 wells were used. In T25 flasks, 2.5×10^6 cells were added while in 6 and 24 well plates, 9.5×10^5 and 1.9×10^5 cells were added respectively following counting of the cells with Trypan blue (Sigma). Trypan blue was used to ensure the right number of viable cells were available for the stimulations per experiment.

Cells were plated 24 hours prior to stimulations so that they reached approximately 70-80% confluency. This was confirmed using the microscope. Cells were typically either treated with 1µg/ml O111:B4 serotype LPS (Sigma), 500µM diclofenac (Sigma) or 10ng/ml IL-4 (R&D Systems) unless otherwise stated. Typically,

in pharmacological experiments, when measuring the dose dependent effect of drugs, a range of concentrations were used and this is stated in the results chapters. Once these stimulants were added, cells were harvested at the end of 24 or 48-hour incubations, unless otherwise stated. To obtain a cell pellet, post-stimulation, the cells were scraped and spun at 500g at 4°C for 5 minutes. The supernatant was decanted and a volume of phosphate buffered saline (PBS) was used to re-suspend the pellet. Cells grown in T25 flasks were re-suspended with 500µl PBS while cells grown in plates were re-suspended with 200µl PBS, to prevent over-dilution. The cells were spun at 500g, at 4°C for 5 minutes and the cell pellet was stored at -20°C.

For experiments where the supernatant contents were measured, the medium post stimulations, were collected in Eppendorf tubes and stored in -80°C until use. The cell pellet was obtained from these samples to standardise supernatant content to protein concentration.

2.1.3 Inhibitory assays

In all experiments a dose response was investigated to identify the optimal concentration of the drug in each experiment. As such, in inhibitory experiments, a 1-hour pre-treatment was typically employed where 0.1µM-10µM SB203580, 100µM-200µM BADGE (Sigma), 1µM-10µM DEX (Sigma) or 5µM-50µM rp-cAMP (Sigma) was added to the cells. The optimal concentrations employed in follow-up experiments were: 10µM SB203580 (Tocris) and 150µM BADGE. These concentrations remained constant unless stated otherwise. Subsequent to this, 1µg/ml LPS, 500µM diclofenac and 10ng/ml IL-4 was added to the inhibitors in medium for either 24 or 48 hours.

In COX-2 activity inhibitory assays, the relevant inhibitor was added at the end of the 24 and 48-hour stimulation. Firstly, at the end of the 24 and 48-hour stimulation, the supernatant was removed with the detached cells and serum-free media was added to the wells for 5 minutes. Serum-free media was added to ensure all the residual diclofenac was washed off. This step was also included in LPS and IL-4 treated cells to maintain consistency in the steps. The plate was placed in the incubator for 5 minutes. Subsequently, this media was removed and the inhibitor, prepared in DMEM immediately before use, was added for 30 minutes. This was followed by the addition of the inhibitor made in medium, with 30 μ M arachidonic acid, also prepared in DMEM, for 15 minutes. The supernatant was collected and immediately stored in -80°C until further analysis.

2.2 MTT assay

It is a common side effect that some drugs actuate toxic effects in cells. It is key to have knowledge on the percent of viable cells at the end of each experiment; thus, fathoming the toxicity levels of each stimulant. This ensures that, what is being measured following various stimulations come from a similar number of cells. Reduction of tetrazolium compounds is used to detect cell viability. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium is positively charged and can penetrate viable eukaryotic cells (Riss et al., 2004). MTT is a measure of mitochondrial activity (Berridge and Tan, 1993). Viable cells convert MTT into formazan, visualised with a purple colour. Formazan is an insoluble precipitate within cells which can be solubilised with dimethyl sulfoxide (DMSO) prior to the absorbance being read at 570nm. The solubilising agent should not interfere with colour stability or induce evaporation (Denizot and Lang, 1986). Dead

cells are incapable of converting MTT to formazan therefore, the colour change is not visible.

Following on from cell stimulations as indicated in the relevant results chapters, 10µl of 5mg/ml (prepared in PBS) MTT (Sigma) was added to 100µl medium in each well. This was incubated for 2 hours at 37°C, 5% CO₂. The contents of the wells were removed and 100µl DMSO was added to each well and mixed thoroughly. The plate was covered in foil until the absorbance was measured at 570nm. The MTT assay was carried out 4 times to determine whether any of these compounds have toxic or proliferative effects on the macrophage J774.2 cell line.

2.3 Bradford assay

Once cells were stimulated, prior to any assay, the protein concentration needed to be quantified. This was done using the Bradford assay (Bradford, 1976). The Coomassie Brilliant Blue G-250 in the Bradford reagent, binds to proteins. Non-covalent Van der Waals force of attraction and electrostatic interaction between the dye and the carboxyl and amino group of the protein respectively were formed. pH<0 shows a red colour at a maximum absorption of 470nm whereas a pH of approximately 1 has a green colour 620nm absorption. At 595nm, a pH>2 shows a bright blue colour. Binding of the protein to the dye induces a shift in maximum absorption from 465nm to 595nm. This method of protein quantification is stable for 1 hour thus making it a good choice. Cations such as sodium and carbohydrates have little to no interference with the binding and therefore the absorbance is unaffected.

Cells were lysed using a mammalian cell lysis kit (Sigma) with protease inhibitor cocktail (Sigma). Following copious preliminary experiments, it was concluded that a total volume of 100µl and 250µl cell lysis buffer was quintessential for cell lysis obtained from a 24-well plate or T25 flask respectively. The cell pellets stored at -20°C (see 2.1) were re-suspended in either 100 or 250µl cell lysis buffer, depending on the starting number of cells. This was left on a shaker on ice for 15 minutes, as per manufacturer's guide. Following this, samples in cell lysis buffer were added to 4x sample buffer (Biorad) with dithiothreitol (DTT) (Biorad) at a 3:1 ratio to make a total volume of 200µl. The remaining sample in cell lysis buffer was used to measure protein concentration.

Tris buffer was used to dilute the bovine serum albumin (BSA) standards from 0.05mg/ml-0.5mg/ml protein. The samples were diluted in tris buffer between 1:2 and 1:5 dilutions. Ten microliters of diluted samples and the standards were added to each well in a 96 well plate in duplicates. Following this, 200µl Bradford reagent (Biorad) was added to each well and left on a shaker for 5 minutes as per manufacturer's instructions. The absorbance was measured at 595nm and a colour gradient could be observed where brown colour indicates low protein concentration and a blue colour indicates high protein concentration. The concentration of protein was quantified using the BSA standards by extrapolating the absorbance against the known concentrations of the standards.

2.4 SDS-PAGE and Immunoblotting

Cells were grown in T25 flasks and stimulated for the appropriate time course. For SDS-PAGE analysis of cell lysates, cells were scraped and then spun (see 2.1) before being stored at -20°C until use. The pellet was re-suspended in cell lysis buffer for 15 minutes on ice (see 2.3). A proportion of this content was used to assay for protein concentration using Bradford reagent (Bio-Rad) (see 2.3); while the remaining was added to 4x sample buffer (Bio-Rad) containing DTT at a 3:1 ratio. The samples in sample buffer were placed in a thermomixer and heat blocked for 5 minutes at 100°C to denature the proteins.

The protein samples were separated by SDS-PAGE using the Mini-PROTEAN 3 gel electrophoresis system (Bio-Rad). The resolving gel was prepared immediately before use, using 3.33ml protogel 30%, 2.5ml 4x resolving buffer, 4.06ml deionized water, 1µl of 10% ammonium per sulfate (APS) per 100ml and 0.1ml tetramethylethylenediamine (TEMED) per 100ml solution. Once the resolving gel solidified, the stacking gel was prepared using 1.3ml protogel, 2.5ml protogel stacking buffer, 6.1ml deionized water, 0.05ml 10% APS and 0.01ml TEMED, and added on top of the resolving gel with 10 well combs. The gels were placed in the tank and filled with running buffer. Twenty microgram proteins were loaded on the 10% polyacrylamide gels.

These were run in running buffer at 100 V, until the dye front reached the bottom of the gel. The running buffer constituted of 25mM Tris, 25mM glycine and 0.1% SDS. To determine the protein size and follow the progress of protein separation, pre-stained Precision Plus Protein Standard (Bio-Rad) was used. This shows molecular

weight in the sizes: 250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa.

After SDS-PAGE, proteins were transferred from gels to 0.2µm pore size polyvinylidene difluoride (PVDF) (G E Healthcare) using the wet transfer method. The transfer buffer constituted of 24mM tris base, 194mM glycine and 10% methanol made in water. A filter sandwich method was used where at the cathode end, a sponge was placed followed by cellulose blot paper (Bio-Rad). On top of this, the SDS-PAGE gel was placed with PVDF that had been activated with 100% methanol. Finally, another cellulose blot paper was added followed by a sponge at the anode end. This transfer sandwich was assembled whilst totally immersed in transfer buffer. The sandwich was secured in cassettes after air bubbles were removed. Ice blocks were added to prevent the cassette from overheating. The cassette was placed in a Mini Trans-Blot electrophoretic transfer cell (Biorad), and run at 100V for 60 minutes. Membranes containing the transferred proteins were referred to as blots.

Blots were incubated in blocking buffer for 1 hour at room temperature on a plate shaker to reduce non-specific antibody binding. Blocking buffer was prepared using 5% dry skimmed milk and 0.1% BSA in wash buffer, which was made using 20 mM Tris Base, 50 mM NaCl and 1:1000 Triton x-100. Blots were then incubated with an appropriate dilution of primary antibody in blocking buffer over night at 4°C. The primary antibody targets the protein of interest and β-Actin to ensure each well contained the same amount of protein (see 2.10). Blots were washed (3 x 5 minutes) in wash buffer (TBS-Tween). Horseradish peroxidase (HRP)-coupled secondary antibody diluted 1:2000 in TBS-Tween was added for 1 hour at room temperature and left on a plate shaker. Secondary antibodies were used depending on the species in

which the primary antibody was raised (see 2.10). After washing the membranes with wash buffer (3 x 5 minutes), the membrane was visualised using enhanced chemiluminescence (ECL) (Bio-Rad) using a developer (Bio-Rad).

Following 5-minute incubations with ECL, blots were placed in a gel imaging system (Chemidoc). Initially, the ladder was scanned using a colometric auto-exposure setting to ensure the band analysed is of the correct molecular weight. Secondly, a high sensitivity setting was used to scan for specific protein bands between 1 and 300 seconds of exposure.

2.5 Enzyme immunoassay (EIA) for PGE₂ quantification

At the end time point for the cell stimulations, serum-free media was used to wash the wells of the 24 well plate. This was incubated at 37°C, 5% CO₂ for 10 minutes. The media was aspirated and 30µM arachidonic acid (Sigma) was added to each well and incubated for 15 minutes (see 2.1). The plate was placed on ice to collect the media into Eppendorf tubes and stored in -80°C until the day of analysis. The plates were stored in -20°C until the Bradford assay was conducted (see 2.3). In inhibitory assays, after serum-free media was used to wash the wells, the appropriate concentration of inhibitor was added to each well for 30 minutes. This was followed by the simultaneous addition of the inhibitor with 30µM arachidonic acid for 15 minutes (see 2.1). At the end of this incubation, the media and plate was stored as mentioned above.

A PGE₂ ELISA kit (Cayman Chemicals) was used to quantify PGE₂ synthesis following each stimulation. Competition for the PGE₂ antibody between PGE₂ in the

samples and PGE₂ acetylcholinesterase (AChE) conjugate (PGE₂ tracer), which is constant in all the wells, determines the concentration of PGE₂ in each treatment group. Thus, the PGE₂ concentration is inversely proportionate to the amount of PGE₂ AChE conjugate that binds to the antibody. The antibody bound PGE₂ can bind to the goat anti-mouse IgG antibody coated in each well. Ellman's reagent contains AChE substrate which allows a colour change to be observed at 405nm after 1 hour of incubation.

Initially, standards from 15.6pg/ml to 2000pg/ml were made via a 1:2 serial dilution in EIA buffer. This was prepared using Ultra-pure water. Each tube containing the standards were vortexed thoroughly to ensure accuracy. The experimental samples were diluted between 1:10 to 1:50 in EIA buffer and vortexed to mix well.

Using the strip 96 well plate provided, the samples were loaded. In the first well nothing was loaded and referred to as 'blank'. This was followed by the non-specific binding (NSB) well, where 100µl EIA buffer was added with 50µl PGE₂ tracer. One well contained 50µl EIA buffer, 50µl tracer and 50µl antibody. For the remaining wells, 50µl of the standards or samples were added followed by 50µl tracer and 50µl antibody. This was left on the plate shaker for 1 hour. Wash buffer was prepared (as per manufacturer's instructions) and the wells were washed 5 times. After each wash, the wells were dabbed on paper towel to remove any wash buffer residue. Ellman's reagent was prepared by reconstituting the provided vial with 20ml ultra-pure water immediately before use. Once 200µl Ellman's reagent was added, the plate was covered in foil and left on a shaker for 1 hour. The absorbance was measured at 405nm.

To calculate the PGE₂ levels per sample, the NSB value was subtracted from B₀. This is referred to as the corrected binding. The NSB absorbance was deducted from absorbance values for standards and samples and divided by the actual binding and then multiplied by 100 to convert to percentage binding. This percentage binding value for the samples was calculated on Graphpad Prism using Log standard concentration and the percentage binding of the standards. The anti-log of these values gave the concentration of the samples. The concentration was multiplied by the dilution factor and divided by 1000 to express the PGE₂ levels in ng/ml. Dividing this value by the protein concentration in the same samples allows the PGE₂ levels to be expressed in ng/mg protein.

2.6 cAMP quantification

cAMP levels following stimulations were measured using ELISA (Cell Signalling). Fifty microliters of sample, standards ranging 0nM-240nM and HRP-linked solution were added to each well. This was covered and left on a horizontal orbital plate shaker for 3 hours at room temperature. cAMP from the supernatant competed with HRP-linked cAMP to bind to the immobilised anti-cAMP antibody. The plate was washed 4 times to remove excess cAMP. Hundred microliters of the chromogenic substrate, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate, was added to each well for 30 minutes at room temperature. This detects HRP activity and yields a blue colour which is inversely proportionatal to the amount of cAMP present in the supernatant. Hundred microliters stop solution was added and the absorbance was measured at 450nm. Sulfuric acid stop solution is a mineral acidic solution that serves to terminate HRP enzymatic activity whilst also stabilising the oxidised products. Addition of this produces a yellow colour of different intensities depending on cAMP concentration.

2.7 PPAR γ transcription factor activity assay

Nuclear extractions were carried out on pellets made from the stimulations which were stored in -20° (see 2.1). Pre-extraction buffer containing DTT and protease inhibitor (AbCam) was used to resuspend the pellet and left on ice. The contents were vortexed and spun 500g, 4°C for 5 minutes. The pellet was resuspended with extraction buffer containing DTT and protease inhibitor cocktail in a 1:1000 dilution. The supernatant was used to measure the activity of PPAR γ transcription factor via ELISA (AbCam).

Complete transcription factor binding assay buffer (CTFB) was prepared as instructed in the manual and 90 μ l of this was added along with 10 μ l nuclear extract to each well. The plate was sealed and incubated overnight without agitation. The wells were emptied the following day and washed 5 times with wash buffer. After each wash, the plate was dabbed on a paper towel to remove contents. Primary antibody that was diluted 1:100 was added to each well, except the blank and incubated for 1 hour at room temperature without agitation. The plate was washed 5 times with wash buffer to remove unbound antibodies. Goat anti-rabbit HRP conjugated secondary antibody diluted 1:100 was added to each well except the blank. This was incubated for 1 hour at room temperature without agitation. The plate was washed 5 times before adding 100 μ l transcription factor developing solution for 15-45 minutes. This was protected from light by covering the plate with foil. A blue colour formed which is proportionate to the activity of PPAR γ in each sample. One hundred microliters stop solution was added to each well to stop the reaction and a yellow colour formed. Absorbance was measured at 450nm. To calculate the PPAR γ activity against the protein concentration, a similar calculation method was followed as mentioned previously (see

2.5). The readings were divided by the calculated protein concentration.

2.8 Cytokine profile ELISA

TNF α and IL-10 levels were quantified with quantitative sandwich EIA (R&D Systems) using supernatants stored at -80°C. Plates purchased from R&D systems were coated with monoclonal antibodies for TNF α or IL-10. Standards were prepared from concentrations ranging from 15.6pg/ml-1000pg/ml for IL-10 ELISA and 10.9pg/ml-700pg/ml for the TNF α ELISA. Fifty microliters standards and undiluted samples were added to each well so the cytokine of interest can bind to the immobilised antibody. The plate was sealed with adhesive tape before placing it on a shaker for 2 hours at room temperature. The contents were aspirated and the plate was washed 4 times to remove all unbound substances. On the final wash, the plate was dabbed on a paper towel to remove excess wash buffer. One hundred microlite conjugated polyclonal antibody for mouse TNF α /IL-10 was added to each well and left on a shaker for 2 hours at room temperature. This was washed with wash buffer 4 times before adding 100 μ l of prepared substrate solution to each well for 30 minutes at room temperature; protected from light. This reaction produces a blue colour. Stop solution provided in the kit was added which allowed the wells to turn yellow. The colour intensity is proportionate to the amount of cytokine bound in the first step. This was read in a spectrophotometer at 450nm. All concentrations were divided by the protein concentration obtained from the Bradford Assay (see 2.3), to standardise the readings.

2.9 Statistical analysis

Once column graphs were generated using Graphpad Prism, the One-way

analysis of variance (ANOVA) was employed to measure any statistical difference between the average of the different groups; where more than 2 conditions were involved. This method of analysis was typically adopted when measuring the effect of multiple concentrations of a stimulant or drug on a particular occurrence.

Similarly, the unpaired T test was used to measure the mean between 2 groups. This was typically between a negative and positive control or vehicle control. Dunnett's post hoc test was performed after the ANOVA to scrutinise deeply, whether the mean from each group was statistically different to the control group, thus the Dunnett's post hoc test is a many-to-one comparison analysis.

Any statistical difference between the groups tested were represented with a symbol ranging from *, \$ and #. The representation for each of the respective symbol is explained in the legend of each figure. If the p value was less than 0.05, it was an indication that the results were not due to chance factors, therefore, the null hypothesis was rejected. Contrariwise, when the p value was greater than 0.05, the null hypothesis was accepted as there was evidence that chance factors played a role in the acquired results. Error bars were representative of the variation in the data supplied and these would interfere with the statistical output.

2.10 Antibodies used in experiments:

<u>Experiment</u>		<u>Antibody</u>	<u>Species</u>	<u>Dilution</u>	<u>Manufacturer</u>
Western blots	Primary Antibody	COX-2	Rabbit	1:1000	Cayman Chemicals
		β -actin	Mouse	1:10,000	Sigma
		Phospho-p38	Rabbit	1:1000	Cell signalling
	Secondary Antibody	HRP-conjugated anti-Mouse	Goat	1:10,000	Cambridge Bioscience
		HRP-conjugated anti-Rabbit	Goat	1:10,000	Cambridge Bioscience
PPAR γ transcription factor activity assay	Primary Antibody	PPAR γ	Rabbit	1:100	Abcam
	Secondary Antibody	HRP-conjugated Anti-rabbit	Goat	1:100	Abcam

Chapter 3: Induction of COX-2 *in vitro* with temporal variations

3.1 Induction of COX-2 by LPS and diclofenac with temporal variations

There are 2 pools of COX enzymes: COX-1, the constitutive enzyme and COX-2, the inducible enzyme. COX are enzymatic proteins that are responsible for prostanoid synthesis. NSAIDs function by blocking COX-1 and COX-2 and therefore, PG production. These drugs have analgesic, antipyretic and anti-inflammatory properties. Despite the therapeutic effects, NSAID usage elicits a wide range of toxic side effects (Hoppmann et al., 1991, Katori et al., 1998, Kotilinek et al., 2008).

Some *in vivo* work has given evidence for NSAID usage at an earlier time point to be beneficial, but at later time points, to exacerbate the inflammatory response (Gilroy et al., 1999, Chan and Moore, 2010). This shows time of NSAID administration to be key. An *in vitro* protocol has been developed where LPS induces COX-2 protein much earlier than diclofenac induces COX-2 (Simmons et al., 1999, Ayoub et al., 2009). The two-time points that were highlighted in this study were 24 hours for LPS induced COX-2 and 48 hours for diclofenac inductive COX-2 (Ayoub et al., 2009). Despite this, it is well established that LPS can induce COX-2 much earlier than 24 hours (Fu et al., 1990, Gilroy et al., 1999, Simmons et al., 1999, Kojima et al., 2000, Eliopoulos et al., 2002, Ayoub et al., 2009). In some *in vivo* experiments, an early and late induced COX-2 protein has been established (Gilroy et al., 1999, Bystrom et al., 2008). Both the early and late induced COX-2 protein has been related to opposing properties such as differential inhibition by acetaminophen and NSAIDs (Simmons et al., 1999, Ayoub et al., 2009). In an attempt to replicate previous data published by Simmons et al. (1999) and Ayoub et al., (2009), and to validate this *in vitro* system in

house, J774.2 macrophages were stimulated with LPS or diclofenac over the 2-time points previously accentuated: 24 hours and 48 hours.

Similar to the protocol used by Ayoub *et al.*, (2009), J774.2 cells were plated in T25 flasks and stimulated with 0.1, 1 and 10µg/ml LPS and 125, 250 and 500µM diclofenac for 24 and 48 hours (Ayoub *et al.*, 2009). A cell pellet was obtained via centrifugation (see 2.1). The cell pellet was lysed and protein concentration determined using the Bradford assay (Bradford, 1976) (see 2.3). Twenty micrograms of protein were run on gels at 100V before being transferred onto 100% methanol activated PVDF membranes. Anti-COX-2 antibody that was diluted 1:1000 in blocking buffer was added to the membranes overnight followed by HRP-conjugated anti-rabbit antibody diluted 1:2000 in blocking buffer (see 2.10). ECL was added to the membranes for 5 minutes before developing (see 2.4).

In order to evaluate COX-2 activity, at the end of the 24 and 48-hour stimulation of cells with LPS and diclofenac, the wells were washed and 30µM AA was added for 15 minutes. The supernatant was collected and assayed for PGE₂ (see 2.5). The absorbances were standardised against the respective protein concentration which was measured using the Bradford Assay (see 2.3)

As expected, LPS induced COX-2 much earlier (fig 3.1.1a) than 500µM diclofenac which induced COX-2 expression at 48 hours (fig 3.1.1b). Diclofenac did not induce COX-2 at 24 hours. The intensity of the 72kDa COX-2 band expression was somewhat similar to the expression of COX-2 induced by LPS at 24 hours. β-

Actin was used as a loading control to ensure the same amount of protein was loaded per well.

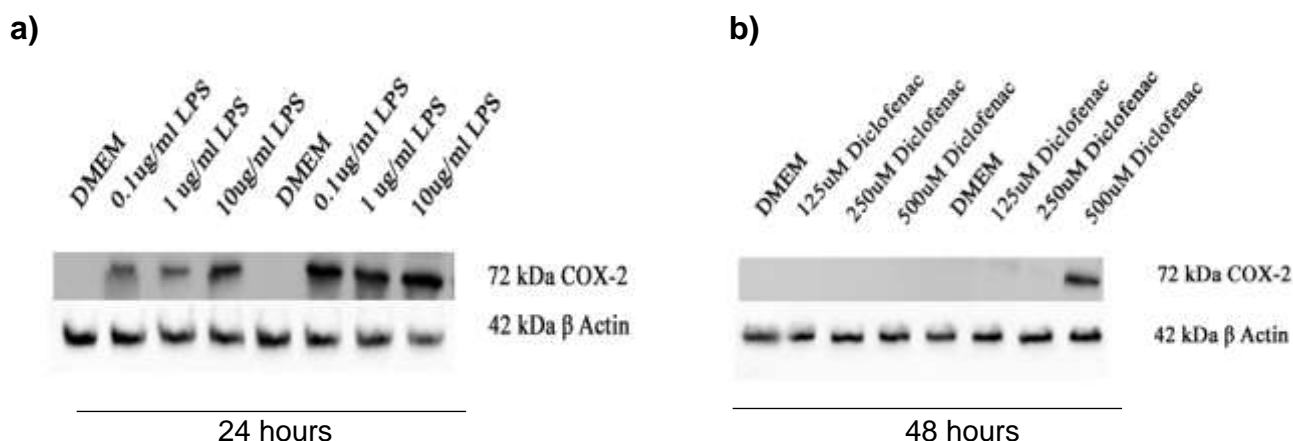


Figure 3.1.1: Induction of COX-2 expression with increasing concentrations of a) LPS and b) diclofenac.

Cells were treated with increasing concentrations of LPS and diclofenac for 24 and 48 hours. Cell pellets were lysed and analysed for protein concentration via Bradford Assay. On 10% gels, 20μg protein was loaded. Subsequent detection with ECL showed the COX-2 band at 72kDa. DMEM represents the unstimulated cells. β Actin was used as a loading control to ensure the same amount of protein was loaded per well. The data shown were obtained from 3 independent experiments (n=5)

LPS induced PGE₂, as a measure of COX-2 activity, in a concentration dependent manner at 24 hours (13.33 ng/mg protein; p<0.05*, 47.33 ng/mg protein; p<0.01** and 328.68 ng/mg protein; p<0.05* ng/mg protein) compared to the negative control (5.54 ng/mg protein) (fig 3.1.2a). At 48 hours, COX-2 was catalytically induced by 10μg/ml LPS (19.28 ng/mg; protein P<0.01**) but not 0.1 and 1μg/ml LPS (10.48 ng/mg protein, 12.7 ng/mg protein) compared to the negative control (5.81 ng/mg protein) (fig 3.1.2a). Increasing concentrations of diclofenac (125, 250 and 500μM) treatment for 24 hours did not induce COX-2 activity (8.07 ng/mg protein, 20.92 ng/mg protein, 5.36 ng/mg protein) (fig 3.1.2b). At 48 hours, 500μM diclofenac significantly induced COX-2 activity (277.12 ng/mg; protein p<0.01**) but not at the other

concentrations (10 ng/mg protein, 25.45 ng/mg protein) compared to the negative control (5.81 ng/mg protein) (fig 3.1.2b). This reinforces data shown by (Simmons et al., 1999) and (Ayoub et al., 2009), where 500µM diclofenac significantly induced late COX-2 activity at 48 hours.

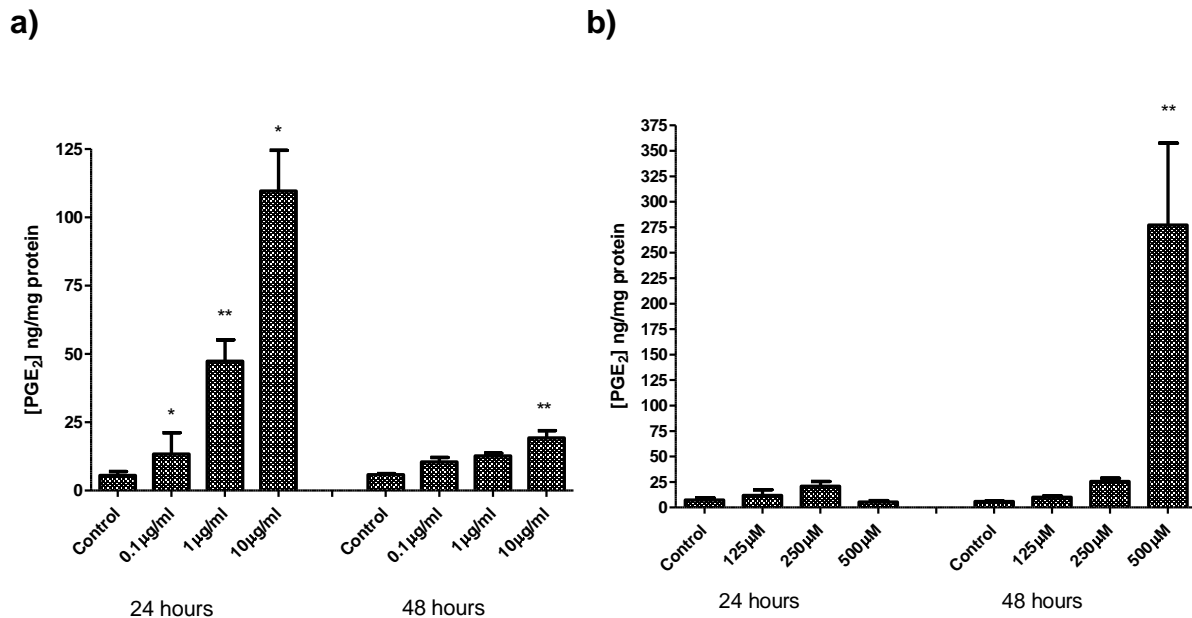


Figure 3.1.2: Induction of COX-2 activity via a) LPS at 24 hours with only 10µg/ml LPS inducing activity at 48 hours. COX-2 activity was induced by b) high concentrations of diclofenac at 48 hours.

Supernatant from cells treated with increasing concentrations of LPS and diclofenac were assayed for PGE₂. All readings were standardised to protein concentration following the Bradford Assay. The negative control contains untreated cells and all consecutive bars reflect increasing concentrations of either LPS or diclofenac at both 24 and 48 hours.

The data shown were obtained from 3 independent experiments (n=3) Statistical significance was determined by One-way ANOVA followed by the Dunnett's post-hoc test. Data are expressed as mean± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001.

3.2 Induction of COX-2 by an anti-inflammatory cytokine, IL-4

Our group hypothesised that the phenotype of the macrophage specifies the functional role of the COX-2 protein. There are 2 types of macrophages, the M1 and M2 macrophages which are pro-inflammatory and anti-inflammatory in nature

respectively (fig 1.2.1.1), although debates still continue about the heterogeneity of macrophage populations in tissues (see for review: (Geissmann et al., 2010)). The M1 macrophage expresses and secretes pro-inflammatory markers and cytokines, whilst activating pro-inflammatory transcription factors. This is antipodal to the M2 macrophage where there is activation of anti-inflammatory transcription factors, expression and secretion of anti-inflammatory markers and cytokines respectively (see for reviews: (Tugal et al., 2013, Liu et al., 2014)). Previously published work show resolution phase macrophages to express COX-2 and produce anti-inflammatory cytokines e.g. IL-10 and TGF β (Bystrom et al., 2008, Ayoub et al., 2009). This along with work showing an exacerbated inflammatory response when COX-2 is inhibited (Gilroy et al., 1999) enables speculation of an anti-inflammatory macrophage to express COX-2.

There is profuse literature depicting the significant difference in the induction pathway of the M1 and M2 macrophage (see 1.2). Extensive research has shown the anti-inflammatory cytokine, IL-4 to be a prototypical activator of the M2 macrophage (Sheldon et al., 2013) via activation of STAT6 (see for reviews: (Lawrence and Natoli, 2011, Hoeksema et al., 2012, Tugal et al., 2013)). IL-4 has been reported to induce STAT6 causing C/EBP β to bind the IL-4 response element of Arg-1, thus activating it (Sheldon et al., 2013). High Arg-1 is an indication of the M2 phenotype (Jimenez-Garcia et al., 2015). Thus, as a next step in this project, whether IL-4 can induce COX-2 expression and activity was investigated. This will enable us to deduce IL-4 as an endogenous inducer of COX-2. To bring together the association between macrophage polarisation and induction of a potentially anti-inflammatory COX-2, whether the two pathways are linked needs to be determined.

Cells were plated in T25 flasks for 24 hours. Flasks were stimulated with 5, 10, 25, 50 and 100ng/ml IL-4 for 24 and 48 hours. Following stimulations, cell lysis buffer was used to lyse the cells (see 2.3). Anti-COX-2 antibody was added to the PVDF membranes and secondary antibody was added the next day (see 2.4).

The anti-inflammatory cytokine, IL-4, induced COX-2 expression specifically at 48 hours with no bands present at 24 hours (data not shown) (fig 3.2.1). The expression of COX-2 was not concentration dependent as the expression did not correlate to the concentrations of IL-4. The negative control, where cells were unstimulated, showed no COX-2 expression as expected. β -Actin expression was used as a loading control.

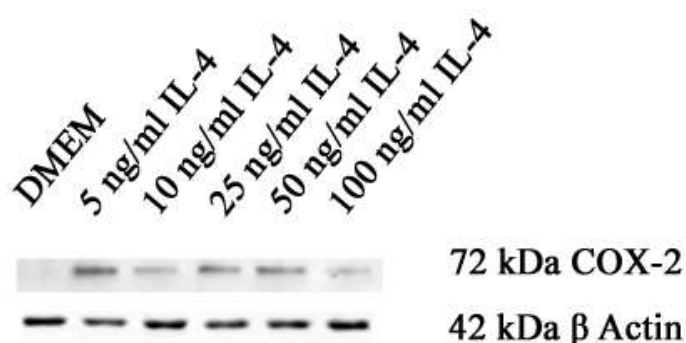


Figure 3.2.1 Induction of COX-2 expression with increasing concentrations of IL-4 at 48 hours.

Increasing concentrations of IL-4 ranging 5-100ng/ml was used to treat cells for 48 hours. Cell pellets were obtained and lysed prior to protein quantification via Bradford Assay. On 10% gels, 20 μ g protein was loaded and anti-COX-2 antibody was used to target COX-2 protein expression. β Actin was used as a loading control. The data shown were obtained from 3 independent experiments (n=3).

Using the same stimulations from the above experiments, ELISA was performed to measure PGE₂ (see 2.5) following stimulations with IL-4. Low concentrations of IL-4: 5, 10 and 25ng/ml were used in this experiment as they were

shown to induce COX-2 protein expression (fig 3.2.1). At the end of the 48-hour stimulation, the cells were washed and treated with 30 μ M AA (see 2.1). Cell pellets were obtained and quantified for protein concentration to standardise all the readings (see 2.3).

IL-4 induced COX-2 activity was analysed at both 24 and 48 hours. The lower concentrations of IL-4 (5, 10 and 25ng/ml) were used in this experiment as they were shown to induce COX-2 protein expression (fig 3.2.1). The higher concentrations of IL-4 were not included as they followed the same pattern of failing to induce COX-2 activity. Whereas 1 μ g/ml LPS induced COX-2 activity at 24 hours (51.63 ng/mg protein; $p < 0.01^{**}$) compared to the negative control (4.16 ng/mg protein), 5, 10 and 25ng/ml IL-4 did not (21.06, 29.09, 23.38ng/mg protein) compared to the vehicle control (8.67ng/mg protein). Activity of COX-2 induced by IL-4 was compared to PBS because IL-4 was reconstituted in this. COX-2 activity was not induced by 500 μ M diclofenac at 24 hours (23.16 ng/mg protein).

COX-2 activity was induced at 48 hours by 1 μ g/ml LPS (35.63 ng/mg protein $p < 0.01^{**}$) and 500 μ M diclofenac (44.36 ng/mg protein; $p < 0.001^{***}$) (fig 3.2.2). Lower concentrations of IL-4 (5 and 10ng/ml IL-4) significantly induced COX-2 activity (44.98 ng/mg protein; $p < 0.001^{***}$, 52.08 ng/mg protein; $p < 0.001^{***}$) but 25 ng/ml did not (2.96 ng/mg protein) (fig 3.2.2). At 48 hours, concentrations of IL-4 greater than 25ng/ml did not induce COX-2 activity (data not shown). At 48 hours, the negative control and vehicle control showed little COX-2 activity (8.01, 14.23 ng/mg protein respectively).

In follow-up experiments, we continued to use 10ng/ml IL-4, which we identified as the optimal working concentration as it induced COX-2 expression and activity. Furthermore, this concentration of IL-4 was previously used by (Bonder et al., 1999, Makita et al., 2015, Ghosh et al., 2016) and was shown to be sufficient to induce an M2 phenotype.

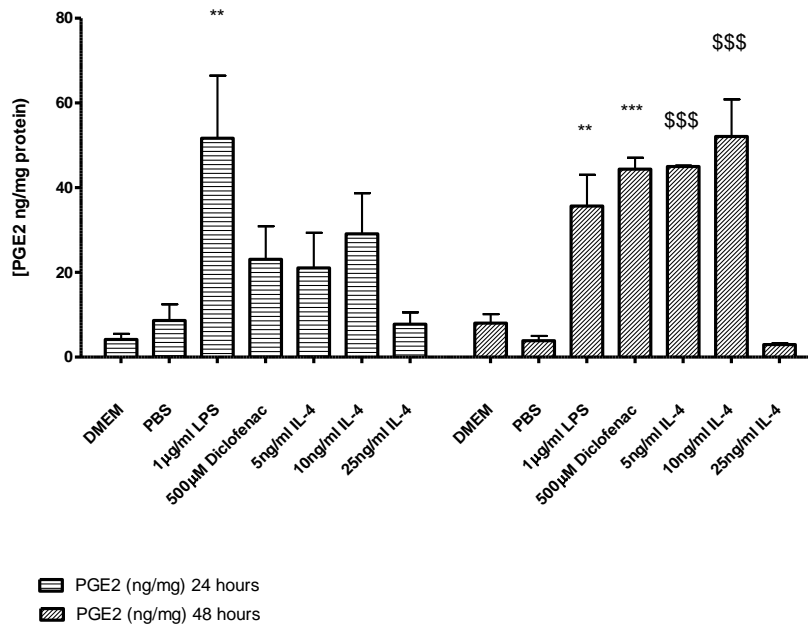


Figure 3.2.2: 5 and 10ng/ml IL-4 induced COX-2 activity at 48 hours but not at 24 hours.

Cells were treated with LPS, diclofenac and increasing concentrations of IL-4 for 24 and 48 hours. At the end of the stimulations, the supernatant was assayed for PGE₂ concentration which was then standardised to its respective protein concentration. The negative control was unstimulated cells while the vehicle control was PBS. The positive control at 24 hours was 1µg/ml LPS and at 48 hours, the positive control was 500µM diclofenac. All other bars represent increasing concentrations of IL-4 treated cells.

The data shown were obtained from 3 independent experiments (n=3). One Way ANOVA was carried out with the Dunnett's post hoc test between the negative control and the vehicle control, represented by * and between the vehicle control and the samples where IL-4 was introduced, represented by \$. Data are expressed as mean±SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, \$p<0.05, \$\$p<0.01, \$\$\$p<0.001.

3.3 Selective inhibition of the late induced COX-2 by acetaminophen

As it can be seen that 10ng/ml IL-4 significantly induced a catalytically active COX-2 protein at 48 hours, further experiments were conducted using 10ng/ml IL-4. NSAIDs hold anti-inflammatory, analgesic and anti-pyretic properties. *In vivo* work has shown that NSAIDs can reduce inflammation if administered in early phases however usage during later stages (possibly the resolution phase) exacerbates inflammation (Gilroy et al., 1999). Treatment of cells for 24 hours with LPS and 48 hours with diclofenac was shown to induce an “early” and “late” COX-2 protein respectively (Simmons et al., 1999) (fig 3.1.1); thus, emulating the bi-phasic COX-2 *in vivo*. This *in vitro* tool was adapted by Ayoub et al. (2009) and also employed in the current study. Simmons et al., (1999) has previously shown LPS, but not diclofenac to induce a COX-2 protein that is insensitive to acetaminophen. This suggests the LPS and diclofenac induced COX-2 to be biochemically varied. Acetaminophen is used as a first line of treatment for pain (see for review: (Botting, 2000, Sharma and Mehta, 2013)). It has been demonstrated as a weak COX inhibitor. As both 500µM diclofenac and 10ng/ml IL-4 induced COX-2 activity at 48 hours, whether acetaminophen can inhibit or reduce IL-4 induced COX-2 activity would be the next appropriate study. To this end, the impact of acetaminophen on LPS, diclofenac and IL-4 induced COX-2 activity was examined.

Cells were plated in 24 well plates for 24 hours to grow. Cells were left unstimulated (negative control) or treated with 1µg/ml LPS, 500µM diclofenac and 10ng/ml IL-4 (positive controls). After 24 and 48 hours, cells were washed with serum free medium for 10 minutes and treated with 0.1, 1, 10 and 100 µM acetaminophen in the acetaminophen control samples and experimental samples. DMEM was added to

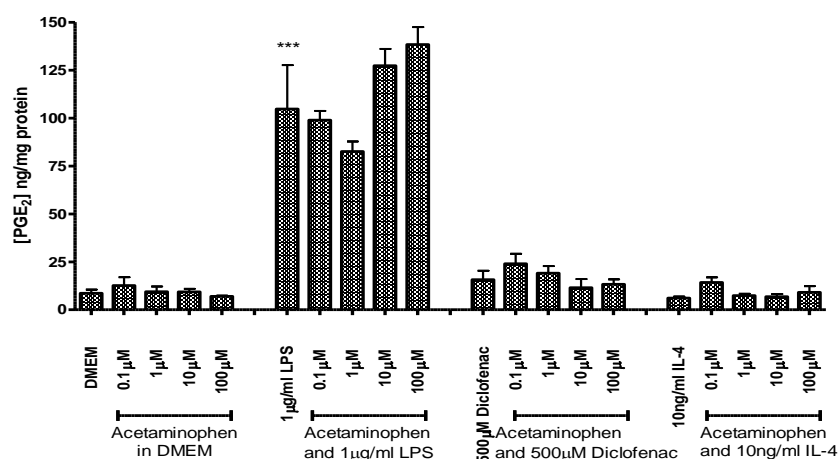
the negative and positive controls. After 30 minutes, cells were treated with 30 μ M AA in all the samples. PGE₂ levels were quantified using the supernatant (see 2.5). Protein concentrations were assayed using the Bradford assay (see 2.3) and the PGE₂ readings were normalised to protein concentration. This range of acetaminophen was adapted from the concentrations used by Ayoub et al. (2009).

As previously shown, LPS significantly induced COX-2 activity at 24 hours (104.76 ng/mg protein $p < 0.001^{***}$). This activity was not increased or decreased in the presence of acetaminophen (98.88, 82.54, 127.31, 138.36 ng/mg protein) (fig 3.3.1a). Neither the unstimulated cells (8.56 ng/mg protein) nor cells treated with acetaminophen alone, induced COX-2 activity (12.61, 9.4, 9.33, 6.97 ng/mg protein). This proves that COX-2 activity in the presence of LPS and acetaminophen is a result of LPS and not the acetaminophen which was introduced. Diclofenac did not induce COX-2 activity at 24 hours (15.71 ng/mg protein) and acetaminophen had no effect on this at 24 hours (24, 19.04, 11.5, 13.24 ng/mg protein). The same was true for IL-4 treatment at 24 hours where, 10ng/ml IL-4 had no effect on COX-2 activity (6.07 ng/mg protein) and acetaminophen did not effect this (14.21, 7.4, 6.83, 9.04 ng/mg protein).

Five hundred micromolar diclofenac and 10ng/ml IL-4 significantly induced COX-2 activity at 48 hours (36.37 ng/mg protein; $p < 0.05^*$, 45.98 ng/mg protein; $p < 0.05^*$ ng/mg protein respectively) (fig 3.3.1b). Only 1 μ M acetaminophen significantly inhibited diclofenac induced COX-2 activity (12.03 ng/mg protein; $p < 0.05^{\$}$) whereas all other concentrations of acetaminophen showed a non-significant inhibition (17.51, 16.28, 19.16 ng/mg protein) (fig 3.3.1b). The highest concentration of acetaminophen inhibited IL-4 induced COX-2 activity (21.3 ng/mg protein; $p < 0.05^{\$}$),

whereas lower concentrations did not show this inhibition (44.9, 38.09, 42.42 ng/mg protein). LPS did not induce COX-2 activity (2.01 ng/mg protein) at 48 hours and acetaminophen did not effect this (3.37, 4.12, 1.76, 2.78 ng/mg protein) (see 3.3.1b). Similar to 24 hours, at 48 hours, acetaminophen on its own did not induce activity of COX-2 in the cells (2.41, 3.89, 4.23, 3.67 ng/mg protein); similar to the unstimulated cells (3.89 ng/mg protein). The diclofenac and LPS data are somewhat similar to data shown by Ayoub et al. (2009).

a)



b)

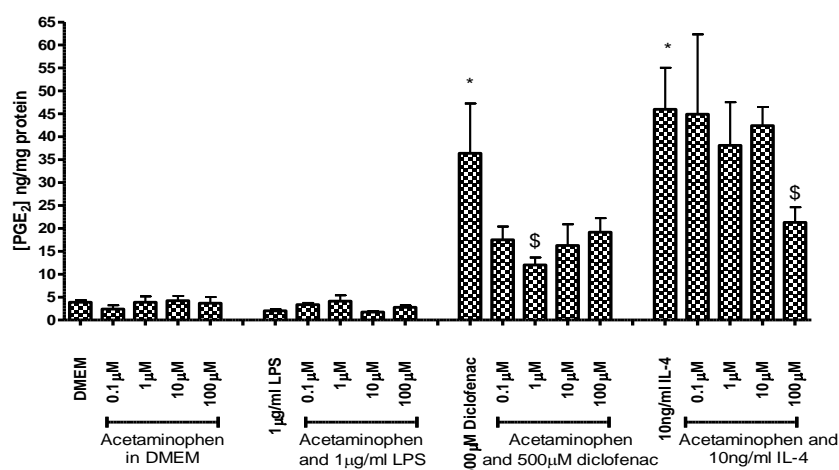


Figure 3.3.1 Graph showing the inhibitory effects of acetaminophen on COX-2 activity at a) 24 hours and b) 48 hours. COX-2 induced by diclofenac and IL-4 were sensitive to inhibition by acetaminophen but LPS induced COX-2 was not. Cells were treated with LPS, diclofenac and IL-4 for 24 and 48 hours followed by a 30-minute treatment with increasing concentrations of acetaminophen. The COX-2 substrate was added before the supernatant was collected for PGE₂ analysis. Readings were divided by the protein concentration which was obtained via the Bradford Assay. The negative control was the unstimulated cells and cells treated with acetaminophen on its own. The positive controls were cells that were treated with only 1 μg/ml LPS, 500 μM diclofenac or 10 ng/ml IL-4. Experimental samples were each stimulant with increasing concentrations of acetaminophen.

The data shown were obtained from 3 independent experiments (n=3). One Way ANOVA was carried out with the Dunnet's post hoc test between the negative control and the positive controls, represented by * and between the positive control and the samples where acetaminophen was introduced with the stimulant, represented by \$. Data are expressed as mean \pm SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, \$p<0.05, \$\$p<0.01, \$\$\$p<0.001.

3.4 Cell viability following stimulations of macrophage J774.2 cells

The MTT assay functions on the principal that the mitochondria dehydrogenase enzyme serves in viable cells. A colorimetric assay enables us to observe a colour change as a measure of cell viability. The diclofenac concentration adopted in this experiment was of a high value: 500µM. There is potential that readings made following diclofenac treatment may relate to the high number of dead cells. As a result, the Bradford assay was conducted after each experiment to normalise all readings to its protein concentration. To this end, having confirmed that diclofenac and IL-4 induced COX-2 are in some ways similar, IL-4 was introduced as a potential endogenous inducer of this protein *in vitro*.

To measure cell viability following the stimulations at the 2 key time points investigated, the MTT assay was carried out. Cells were grown in 96 well plates and treated with either 1µg/ml LPS, 500µM diclofenac or 10ng/ml IL-4 for 24 and 48 hours. At the end of the stimulations, 10µl of 5mg/ml MTT in PBS was added to each well. Following a 2-hour incubation, 100µl DMSO was added to each well and mixed before measuring the absorbance. Readings made at 0 hr were deducted from readings made at 24 and 48 hours to measure the change in viable cell density.

At 24 hours, cells treated with 1µg/ml LPS (1.07nm; $p < 0.05^*$) and 10ng/ml IL-4 (1.1nm $p < 0.05^*$) showed high MTT readings compared to the unstimulated cells (0.41nm) (fig 3.4.1). Diclofenac on the other hand showed low readings (0.3nm). A similar pattern was observed at 48 hours, where high MTT readings were observed in the unstimulated cells (0.8nm) and in cells treated with 1µg/ml LPS (0.98nm) and

10ng/ml IL-4 (1.69nm) (fig 3.4.1). In cells treated with 500μM diclofenac, the reading was extremely low, (-0.69nm; $p<0.05^*$).

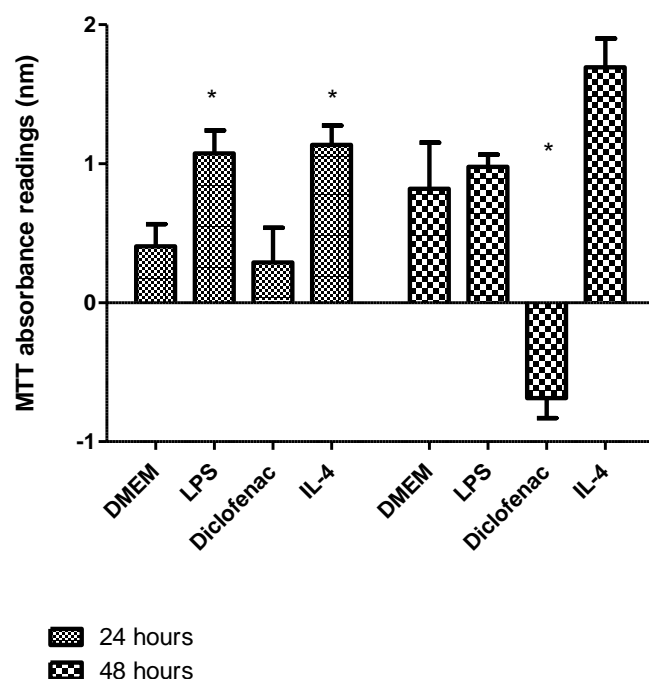


Figure 3.4.1 MTT assay showing high concentrations of diclofenac to reduce cell viability.

Cells were simulated with 1μg/ml LPS, 500μM diclofenac and 10ng/ml IL-4 for 0, 24 and 48 hours before measuring formazan production. The readings made at 0hr were deducted from the readings made at 24 and 48 hours. The negative control in this experiment was the unstimulated cells. All the bars represent cell viability following each stimulation.

The data shown were obtained from 3 independent experiments ($n=3$). One Way ANOVA was carried out between the untreated cells and the treated cells. Data are expressed as mean \pm SEM (error bars); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.5 Discussion to results chapter 3

Induction of COX-2 protein *in vitro* with temporal variations

3.5.1 Induction of an early and late COX-2 by LPS and diclofenac

Although, previously thought to be a single protein, it has now been accepted that there are 2 COX isoforms; one constitutive (COX-1) and one inducible (COX-2). Addition of cycloheximide only blocked the expression of the inducible COX-2 protein (Habenicht et al., 1985, Fu et al., 1990, Xie et al., 1991, Katori et al., 1998). Whereas COX-1 produces cyto-protective PG, COX-2 was believed to play a role in pathological diseases (see for review: (Suleyman et al., 2007)). Inhibition of this protein by NSAID is commonly used for the effective management of inflammation, however, side effects such as GI erosions have been reported (see for review: (Suleyman et al., 2007)). This has been accredited to the inhibition of the constitutive COX isoform. As a result, selective COX-2 inhibitors were developed however, these too reflect adverse effects (see for review: (Kawai, 1998, Suleyman et al., 2007)). It has been suggested that the selectivity of these drugs to COX-2 becomes abolished at high concentrations (Suleyman et al., 2007); however we suggest the nature of the COX-2 expressed to define the response to these class of drugs. We allude to the time of drug administration being key to obtain beneficial or adverse effects. Thus, inhibition of, what we predict to be an 'anti-inflammatory' COX-2, may lead to such adverse reactions.

Firstly, it must be noted whether LPS and diclofenac do indeed induce COX-2 expression at various time points as suggested by Simmons *et al.*, (1999) and Ayoub *et al.*, (2009). This will give a functioning *in vitro* tool which emulates the 2 peaks of

COX-2 expression reported *in vivo* (Gilroy et al., 1999). Further to identifying COX-2 expression at different times, the properties of these should be studied. If there are indeed differences, investigating whether the induction pathways vary for the early and late induced COX-2, *in vitro*, would be the natural next step. The initiator of the induction pathways leading to COX-2 needs to be clarified. Evidence of temporal variations in this induction would mean greater scrutiny in administration of COX inhibiting drugs, as the time at which patients take the drug would need to be controlled/regulated.

Treating the macrophage J774.2 cell line with 1µg/ml LPS or 500µM diclofenac (Ayoub *et al.*, 2009) has been shown to induce COX-2 expression at 24 and 48 hours respectively (fig 3.1.1). COX-2 expression intensified with a concentration dependent increase in LPS at both 24 and 48 hours, however at 24 hours, there was no COX-2 expressed following treatment of cells with 500µM diclofenac. At 48 hours, COX-2 expression was apparent with 500µM diclofenac treatment (fig 3.1.1b). Thus, the data shown in fig 3.1.1 was consistent with previously published data (Ayoub et al., 2009) where COX-2 was induced by 24 hours following LPS stimulations and at 48 hours following stimulations with 500µM diclofenac. The different induction times may allude to the COX-2 protein serving differential purposes. We believe there is a translational block and removal of this may depend on the COX-2 function. This comes from unpublished work which show the mRNA of COX-2 to be expressed much earlier than the expression of the protein (Ayoub, unpublished). Various *in vivo* experiments have shown selective inhibition of the early COX-2 to wane the inflammatory response and inhibition of the late COX-2 to exacerbate inflammation (Gilroy et al., 1999, Chan and Moore, 2010). This therefore suggests the COX-2 induced at both time points to

possess different functions. Though speculations at this stage, the different properties may be dictated by the inflammatory milieu and its effects may be mediated by the downstream PG produced and the receptor to which they bind.

LPS was shown to induce COX-2 activity at 24 hours in a dose dependent manner. This activity dropped by 48 hours where activity was only significantly induced by 10µg/ml LPS; a concentration that is not physiologically relevant (fig 3.1.2). The diclofenac treated cells showed no COX-2 activity at 24 hours, as expected, because at this time point, no induction of the protein was noted (fig 3.1.1). At 48 hours, there was a significant upsurge in PGE₂ production following treatment of cells with 500µM diclofenac (fig 3.1.2). This coincides well with fig 3.1.1 which shows COX-2 expression induced by this concentration of diclofenac. This alludes to the early induced COX-2 to serve a different purpose to the late induced COX-2, as the expression appears to be tightly controlled.

3.5.2 Evidence of macrophages that express COX-2 following IL-4 treatment

Despite IL-4 being an anti-inflammatory cytokine, we gave evidence for COX-2 expression following 48-hour stimulations with increasing concentrations of IL-4 (fig 3.2.1). COX-2 expression was absent at 24 hours. This pattern being somewhat similar to the induction of COX-2 by diclofenac (fig 3.1.1b), leads to the assumption that diclofenac and IL-4 induce a phenotypically similar COX-2 protein, in comparison to the LPS induced COX-2 protein. It may be that the induction pathway is similar between IL-4 and diclofenac. There does not seem to be a concentration dependent effect on the expression of this protein by IL-4, as there is no pattern in the intensity of

the band. The effect of IL-4 seems to be somewhat powerful as low concentrations (5ng/ml) show protein induction (fig 3.2.1). It has previously been reported that IL-4R α is expressed on this cell line (Jimenez-Garcia et al., 2015) therefore, the effect of IL-4 may be immediate. IL-4 binds to IL-4R α with picomolar affinity. This binding recruits IL-2R γ γ_c chain which forms a type 1 receptor complex. Although the IL-4R complex is not endowed with kinase activity, the cytoplasmic tail is linked with the recruitment of JAK. Typically, this activates phosphorylation of STAT6 (see for review: (Mueller et al., 2002, Luzina et al., 2012)).

Following experiments that showed IL-4 to induce COX-2 expression (fig 3.2.1), whether this protein is catalytically active needed to be understood. A catalytically active protein confirms that the induction serves a purpose at that time point. As the negative (DMEM) and vehicle (PBS) control exhibit no COX-2 activity, while the positive controls (1 μ g/ml LPS at 24 hours and 500 μ M diclofenac at 48 hours) produced significantly more PGE₂ (fig 3.2.2), the effect of IL-4 could be compared to the controls confidently.

Supporting data from fig 3.1.2, whereas at 24 hours, LPS treated cells displayed COX-2 activity, IL-4 treated cells did not. Low concentrations of IL-4 increased PGE₂ output compared to the negative control at 48 hours (fig 3.2.2). This is similar to the diclofenac treated cells, thus strengthening the argument that the diclofenac and IL-4 induction of COX-2 may share a similar pathway. Higher concentrations of IL-4 did not induce COX-2 activity and there was a sharp decline in PGE₂ yield. This may be explained by a concept reviewed by Mueller et al. (2002) who suggested a single molecule to play both agonistic and antagonistic roles in signalling pathway via

cytokine receptor inhibition that is induced by high doses of its cognate ligand. This was previously noted in growth hormones where receptor dimerization was halted due to receptor saturation. Receptor saturation was not measured in this project so the same idea cannot be concluded confidently.

Collectively, these figures showed COX-2 protein activation downstream of IL-4 binding its receptor in what was assumed to be an anti-inflammatory signalling pathway. Copious publications have shown IL-4 to polarise macrophages towards M2 as shown by increased Arg-1 in macrophages (Sheldon et al., 2013), activation of STAT 6 (Kaplan et al., 1996) and activation of anti-inflammatory receptors like PPAR γ (Szanto et al., 2010) (see for review: (Luzina et al., 2012)). Furthermore, IL-4 is known to inhibit Th1 differentiation and pro-inflammatory signalling (see for review: (Zamorano J, 2003)). This raises the question as to whether the COX-2 induced by IL-4, is an active member of the anti-inflammatory pathway.

There is evidence that diclofenac induced COX-2 mRNA is present hours before the protein is expressed (Ayoub, unpublished data). Thus, it can be speculated that the IL-4 induced COX-2 mRNA is translationally repressed; leading to the protein being expressed only at 48 hours. From this, it can be inferred that the COX-2 induced by both diclofenac and IL-4 may have different properties to the LPS induced COX-2. There is a chance that the late COX-2 may be translationally inhibited hence the delayed expression.

Translational repression can be tightly regulated by micro RNA (miR). There is profuse literature which show specific miR that control COX-2 translation. miR have

been reported to down-regulate COX-2 protein synthesis and downstream PGE₂ synthesis (Yoon, 2011). miR-16 was shown to repress COX-2 translation without effecting COX-2 mRNA, by binding to the miR-16 response element on the 3'untranslated region (3'UTR) of COX-2 in hepatoma cells (Agra Andrieu et al., 2012). Interestingly, in microglia specifically, miR-124 was shown to be expressed in M2 phenotypes however in disease such as experimental autoimmune encephalomyelitis (EAE), levels of miR-124 were reduced. This correlates with a reduction in the M2 marker, MR, and an increase in M1 markers CD86 and MHC Class II (Ponomarev et al., 2011). The mRNA should remain stable and avoid degradation in order to be translated into a fully functioning protein. Natural mechanisms have evolved to preserve the mRNA such as polyadenylation of the mRNA (see for review: (Ross, 1995)). Thus, there is a possibility that the IL-4 induced COX-2 transcript is preserved until translation is activated.

One of the hypotheses of this study was that macrophages polarise prior to the induction of COX-2 and we believe that the phenotype directs the functional properties of the COX-2. We speculate COX-2 translational postponement to control the properties of the COX-2 expressed (whether pro-inflammatory or anti-inflammatory). COX-2 induced in the late phase has been suggested to be anti-inflammatory as Ayoub *et al.*, (2009) showed IL-10 and TGF β release.

It would be important to study the expression of IL-4R to see whether the stimulus is capable of increasing or reducing this receptor to induce a response. In microglia, LPS was shown to increase IL-4R α in microglia of BALB/c mice (Fenn et al., 2012) however Quentmeier et al. (1994) showed LPS increased IL-4

responsiveness but not expression of IL-4R in human MONO-MAC-6 cells. This suggests a mechanism by which resolution, or at least suppression of a pro-inflammatory response, can be instigated in an attempt to control inflammation (see for review: (Luzina et al., 2012)).

There is clinical evidence of adversities reported from NSAID usage; increasing risk of hypertension, stroke and myocardial infarction (see for review: (Stollberger and Finsterer, 2003)). This project proposed that the adverse effects reported may be caused by the inhibition of the late COX-2 which we suggest to be expressed by macrophages of opposing phenotype to the macrophages expressing COX-2 in LPS treated cells. This “anti-inflammatory” COX-2 may play a role in producing PGs which drive secretion of anti-inflammatory cytokines whilst also reducing secretion of pro-inflammatory cytokines.

3.5.3 Selective inhibition of COX-2 by acetaminophen

Acetaminophen has been shown to specifically inhibit diclofenac induced COX-2 but not the LPS induced COX-2 activity (Simmons et al., 1999, Ayoub et al., 2009). As IL-4 induced COX-2 protein at the same time as diclofenac, whether the two COX-2 proteins are similar needed to be confirmed so that IL-4 could be used as the endogenous inducer for COX-2. Diclofenac is highly toxic to the cells (fig 3.4.1) as shown by lower MTT readings following addition of this drug. The effect of high diclofenac concentrations for a longer duration severely reduced the cell count (fig 3.4.1). If IL-4 could be identified as a late inducer of COX-2, this may be a more pharmacologically relevant tool *in vitro* for COX-2 induction. As a first step, understanding whether IL-4 induced COX-2 is sensitive to acetaminophen, similar to

diclofenac induced COX-2 will enable us to draw further conclusions as to whether IL-4 and diclofenac induced COX-2 are similar. Subsequently, IL-4 should be used to endogenously induce COX-2 protein.

As expected, LPS, diclofenac and IL-4 induced COX-2 activity at the previously shown time points. Acetaminophen treated cells appeared to produce PGE₂ levels, at similar concentrations to what was seen in the unstimulated cells (fig 3.3.1). Thus, any changes in PGE₂ production was dependent on the impact of acetaminophen on the COX-2 induced by the different stimuli.

This experiment further confirmed data provided by Ayoub et al. (2009) who showed LPS induced COX-2 to be insensitive to acetaminophen while diclofenac induced COX-2 activity was reduced by acetaminophen. Diclofenac and IL-4 did not induce COX-2 activity at 24 hours and acetaminophen had no stimulatory effect on this (fig 3.3.1). This supports the notion that acetaminophen is not an inducer of COX-2 activity (fig 3.1.1) (Simmons et al., 1999). As shown previously, COX-2 was induced by diclofenac and IL-4 at 48 hours (fig 3.1.2, 3.1.4, 3.1.5). Low concentrations of acetaminophen were able to inhibit diclofenac induced PGE₂ synthesis but only higher concentrations of acetaminophen (100µM) inhibited IL-4 induced COX-2 activity (fig 3.3.1b). The difference in sensitivity levels suggests the induction of COX-2 varies in some ways between IL-4 and diclofenac. It could be argued that 100µM acetaminophen is too high a concentration because the therapeutic plasma concentration is 10µM, thus firm conclusions from this cannot be made. As 1µM acetaminophen inhibited 500µM diclofenac induced COX-2 activity (Ayoub *et al.*, 2009) and all other concentrations of acetaminophen non-significantly reduced

diclofenac induced COX-2 activity, this points to the diclofenac induced COX-2 to have different biochemical properties to the LPS induced COX-2.

This inhibition is vital as we consider the late COX-2 to be anti-inflammatory and part of the resolution process. Based on work by Ayoub et al. (2009), we suggest the macrophage expressing this COX-2 to be anti-inflammatory therefore any inhibition by drugs would impair the natural healing process. Inhibition of the late COX-2 exacerbates the inflammatory response *in vivo* (Gilroy et al., 1999, Chan and Moore, 2010). If this COX-2 possesses anti-inflammatory and pro-resolving properties, then the ability of acetaminophen to inhibit this COX-2 is detrimental. Acetaminophen being an over the counter (OTC) drug, therefore being easily accessible, further complicates the matter.

Acetaminophen has been recorded and discussed as a toxic drug on several platforms. Although its mechanism of action remains a pharmacological enigma, acetaminophen was suggested to work centrally; whilst being a weak inhibitor of COX-1 and COX-2. (Flower and Vane, 1972). Whereas NSAIDs inhibit COX by competing with AA, acetaminophen caused reduction within the peroxidase site (see for review: (Hinz and Brune, 2012)). Flower and Vane (1972) showed brain PG to be more sensitive to acetaminophen inhibition compared to spleen PG. This was suggested to be due to impaired acetaminophen inhibition of PG caused by high extracellular AA and presence of peroxide in inflamed tissue (Hinz et al., 2008). Efforts were made to suggest acetaminophen as an inhibitor of 'COX-3', a variant of the COX-1 gene; however this notion appears to be rejected (see for review (Hinz and Brune, 2012)); although some pharmacological evidence supported this (Ayoub et al., 2011). Hinz et

al. (2008) reported an average 66% inhibition of LPS induced PGE₂ and coagulation induced TxB₂ in blood from acetaminophen treated patients. COX-1 inhibition was somewhat less compared to COX-2 (Hinz et al., 2008). The short half-life of acetaminophen meant its inhibitory effect on COX-2 was short-lived (see for review: (Hinz et al., 2008)).

Previous reports and case studies have shown acetaminophen to be lethal, commonly used to over-dose (Sheen et al., 2002). The question rises as to whether the toxic effects of acetaminophen are seen due to inhibition of an anti-inflammatory COX-2 protein during the resolution phase.

3.6 Chapter conclusion

There appears to be tight regulation of COX-2 expression which is induced with temporal variation. COX-2 could be induced early or at a later stage depending on the stimulus. The LPS and diclofenac induced COX-2 have differential inhibition by acetaminophen which suggests different biochemical properties between both proteins (fig 3.3.1). Furthermore, IL-4 was shown to induce a catalytically active COX-2 protein specifically following 48-hour treatments, similar to diclofenac (fig 3.2.1, 3.3.2). The next step would be to look into the signalling pathways that induce COX-2 and see how this varies between the different stimulus. This would provide potential drug targets to stimulate the induction of what we predict to be an anti-inflammatory COX-2 protein.

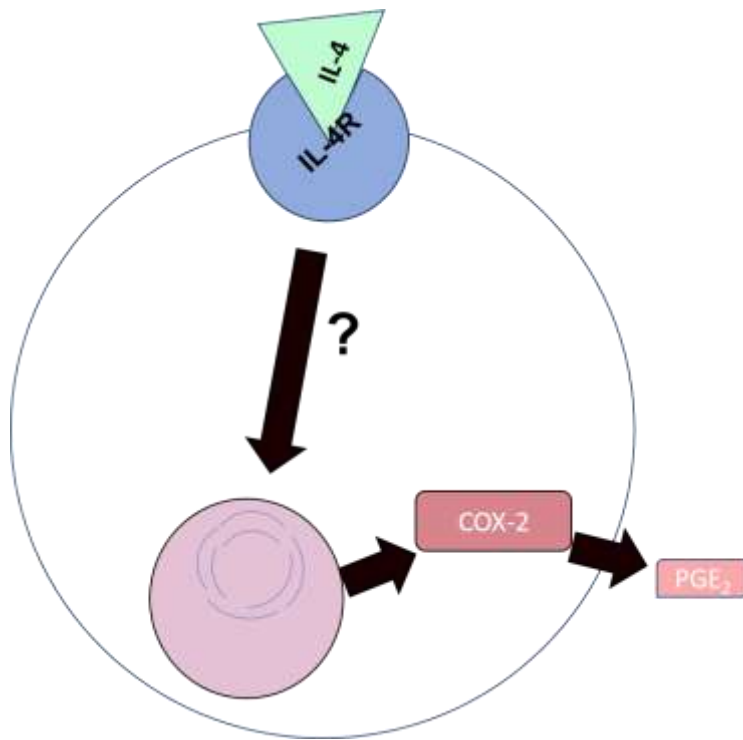


Figure 3.6.1 Diagram to conclude chapter

Treatment of macrophage J774.2 cells with 10ng/ml IL-4 for 48 hours induced the expression of COX-2 that is catalytically active, as shown by PGE₂ production. PGE₂ production was reduced by the addition of 100μM acetaminophen.

Chapter 4: Signalling pathway involved in the induction of COX-2 by IL-4

4.1 PPAR γ is required for the induction of COX-2 by IL-4

PPAR γ is a member of a group of receptors that serve to drive gene transcription by heterodimerising with retinoid-X receptor following ligand activation (Wick et al., 2002). This receptor has been linked with neuroprotection and the curtailing of inflammatory genes (Kapadia et al., 2008). TZDs, which are agonists to PPAR γ , have been used in animal models of neurological disease and CVD (Kapadia et al., 2008) to reduce pro-inflammatory parameters. The fact that PPAR γ is involved in anti-inflammatory pathways (Kapadia et al., 2008), suggest an involvement in a compensatory mechanism during inflammation (Wang et al., 2015).

It would be important to note whether activation of PPAR γ can induce COX-2 expression by using the PPAR γ agonist, rosiglitazone (see for review: (Chiarelli and Di Marzio, 2008)). In order to establish whether PPAR γ is involved in IL-4 induced COX-2, the PPAR- γ antagonist, BADGE, must be used to assess whether this transcription factor is required in the induction pathway. Previously, Ayoub et al., (2009) showed the PPAR γ antagonists, BADGE and GW9662, to block diclofenac induced COX-2 but not LPS induced COX-2. The same protocol was used to pharmacologically assess whether IL-4 induces COX-2 expression and activity via PPAR γ . This experiment may bring us closer to understanding the IL-4-PPAR- γ axis and its involvement in the COX-2 induction pathway.

Cells were plated in T25 flasks for 24 hours. At the end of this, cells were treated with increasing concentrations of rosiglitazone (1, 10, 100 μ M). Rosiglitazone was

dissolved in DMSO; therefore, a vehicle control was used where cells were simply treated with 0.1% DMSO for 48 hours. At the end time point, a cell pellet was collected (see 2.1) and this was analysed for COX-2 and phosphorylated p38 protein expression (see 2.4). p38 MAPK expression was observed to assess whether activation of PPAR γ by rosiglitazone phosphorylates p38.

In a subset of experiments, cells were plated in T25 flasks for 24 hours. Cells were either treated with 100-200 μ M BADGE alone (negative control) or left unstimulated for 1 hour. This was followed by the addition of 500 μ M diclofenac or 10ng/ml IL-4 (positive control) or diclofenac and IL-4 co-treated with 100, 150 and 200 μ M BADGE. The concentrations of BADGE were obtained from Ayoub et al. (2009). At the end of the stimulations, cell lysis buffer was used to lyse the cells and quantify the proteins (see 2.3). In Western blot experiments, the target protein expression was observed (see 2.4).

To investigate whether the addition of BADGE inhibited the activity of COX-2 following the stimulations above, the supernatant was collected (see 2.1). COX-2 activity was measured via ELISA (see 2.5). The working concentration of BADGE used in this experiment was 150 μ M BADGE, as this concentration was found to inhibit COX-2 expression without causing excessive cell death.

The agonist for PPAR γ , rosiglitazone, was shown to induce COX-2 expression as early as 24 hours (fig 4.1.1), whereas the negative control and the vehicle control (DMSO) did not. Phosphorylated p38 was expressed in cells treated with rosiglitazone. With increasing concentrations of rosiglitazone, the intensity of phosphorylated p38

increased (fig 4.1.1); showing a positive relationship. At 48 hours, the intensity of both COX-2 and phosphorylated p38 was greater than at 24 hours. β -actin expression was measured to ensure the same amount of protein was loaded per well.

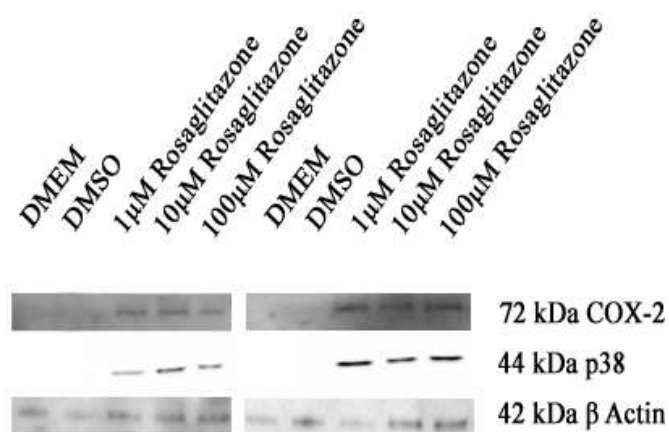


Figure 4.1.1 Rosiglitazone induced COX-2 expression whilst activating P38.

Cells were treated with increasing concentrations of rosiglitazone for 24 and 48 hours. The cells pellet was lysed before loading onto 10% gels. Unstimulated cells were the negative control and DMSO of the greatest percentage was the vehicle control. COX-2, phosphorylated p38 and β Actin expression was observed. The data shown were obtained from 3 independent experiments ($n=3$).

Expression of COX-2 by diclofenac was inhibited with increasing concentrations of BADGE, starting as early as 100 μ M BADGE (fig 4.1.2a). This experiment was carried out at 48 hours, because diclofenac did not induce COX-2 expression at 24 hours. Treatment of cells with BADGE did not induce COX-2 expression in this cell line. The concentrations of BADGE used in this experiment was adapted from Ayoub et al. (2009) to both replicate and confirm the working concentration. Similar to diclofenac, IL-4 treated cells that were co-treated with BADGE, inhibited COX-2 (fig 4.1.2b).

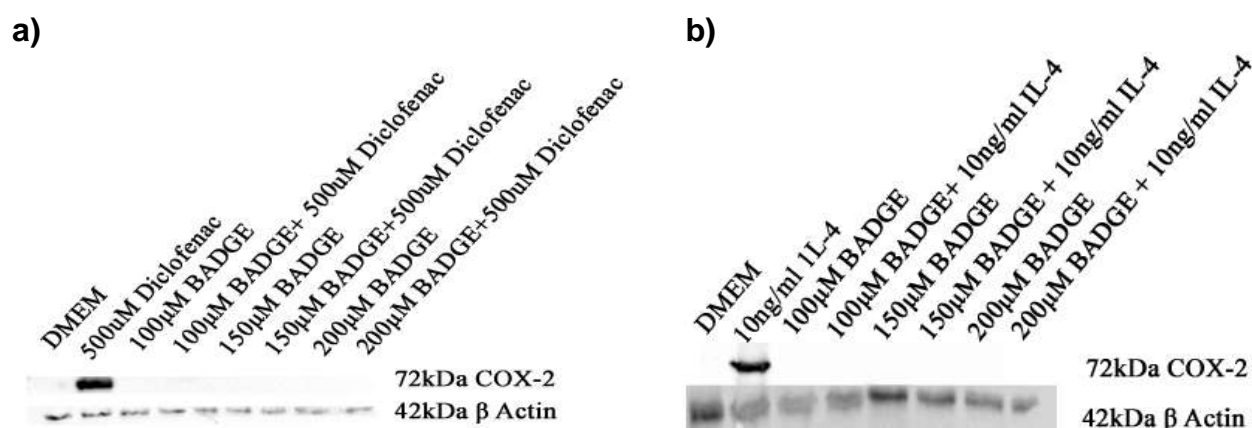


Figure 4.1.2 BADGE completely blocked expression of COX-2 induced by a) diclofenac and b) IL-4 at 48 hours.

Cells were treated with diclofenac or IL-4 with increasing concentrations of BADGE for 48 hours. Post-stimulation, cell pellets were lysed and quantified for protein concentration. COX-2 and β -Actin expression were observed on the membrane. Unstimulated cells and cells treated with BADGE alone were the negative controls. The positive controls were cells treated with either diclofenac or IL-4 alone. The data shown were obtained from 3 independent experiments ($n=3$).

At 48 hours, both diclofenac and IL-4 induced COX-2 activity (48.94 ng/mg protein; $p<0.01^{**}$, 57.79 ng/mg protein; $p<0.01^{**}$ respectively). In the presence of BADGE, the activity was significantly reduced in diclofenac and IL-4 treated cells (15.93 ng/mg protein; $p<0.05^{\$}$, 25.12 ng/mg protein; $p<0.05^{\$}$ respectively) (fig 4.1.3). At 48 hours, LPS did not induce COX-2 activity (22.85 ng/mg protein) and the addition of BADGE did not alter this (19.23 ng/mg protein) (fig 4.1.3). At 48 hours, the negative control and the vehicle control did not induce COX-2 activity (8.08, 21.48 ng/mg protein respectively). BADGE was shown to have no effect on COX-2 activity on its own at 24 hours (2.88 ng/mg protein) compared to the unstimulated cells (5.92 ng/mg protein). The addition of BADGE (82.89 ng/mg protein) had no effect to LPS treated cells (87.04 ng/mg protein) (fig 4.1.3). Diclofenac and IL-4 did not induce COX-2 activity (7.23, 3.99 ng/mg protein respectively) and this was not effected by the addition of BADGE (4, 2.87 ng/mg protein respectively).

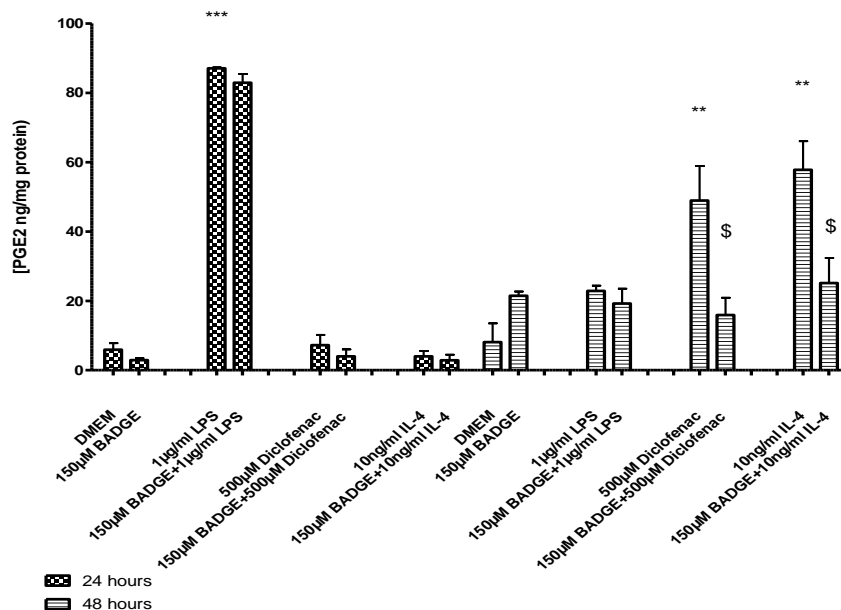


Figure 4.1.3 At 48 hours, BADGE significantly reduced diclofenac and IL-4 induced COX-2 activity but had no effect on LPS induced COX-2 activity.

Cells were treated with LPS, diclofenac or IL-4 for 24 and 48 hours in the absence and presence of BADGE. At the end of the stimulations, the supernatant was assayed for PGE₂. Absorbances were divided by the protein concentration to standardise all the readings. The negative control were unstimulated cells or cells treated with BADGE alone. The positive control were cells treated with either 1µg/ml LPS, 500µM diclofenac or 10ng/ml IL-4 in the absence of BADGE. All other bars represent the effect of BADGE on PGE₂ production by LPS, diclofenac and IL-4.

The data shown were obtained from 3 independent experiments (n=3). One Way ANOVA was used to compare the PGE₂ levels produced between the negative and positive controls, as represented by *, and between the positive control and samples where BADGE was introduced as represented by \$. Data are expressed as mean±SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, \$p<0.05, \$\$p<0.01, \$\$\$p<0.001.

4.2 Induction of PPAR γ by LPS and IL-4

To understand whether PPAR γ is differentially activated by LPS and IL-4, the activity of this transcription factor was investigated. Cells were grown in 6 wells plates for 24 hours, before being treated with 1µg/ml LPS or 10ng/ml IL-4. These stimulations were terminated at the following time points: 30 minutes, 2, 6, 24, 36 and 48 hours. Nuclear extraction was carried out on the cell pellet before being analysed for PPAR γ activity via a PPAR γ transcription factor assay kit (Abcam) (see 2.7).

At all the time points investigated, there was basal PPAR γ activity (3.79, 5.78, 2.07, 1.56, 2.94, 1.2 ng/mg protein) (fig 4.2.1). Specifically at 24 and 36 hours, 1 μ g/ml LPS reduced PPAR γ activity compared to the negative control (1.02 ng/mg protein; $p<0.001^{***}$, 1.34 ng/mg protein; $p<0.01^{**}$ ng/mg protein respectively) (fig 4.2.1). At all other time points following LPS treatment, there was no change in PPAR γ activity (3.65, 4.26, 1.51, 1.68 ng/mg protein). PPAR γ activity decreased by 6 hours (2.07 ng/mg protein); with a sudden peak at 36 hours (2.94 ng/mg protein) in the unstimulated cells (fig 4.2.1).

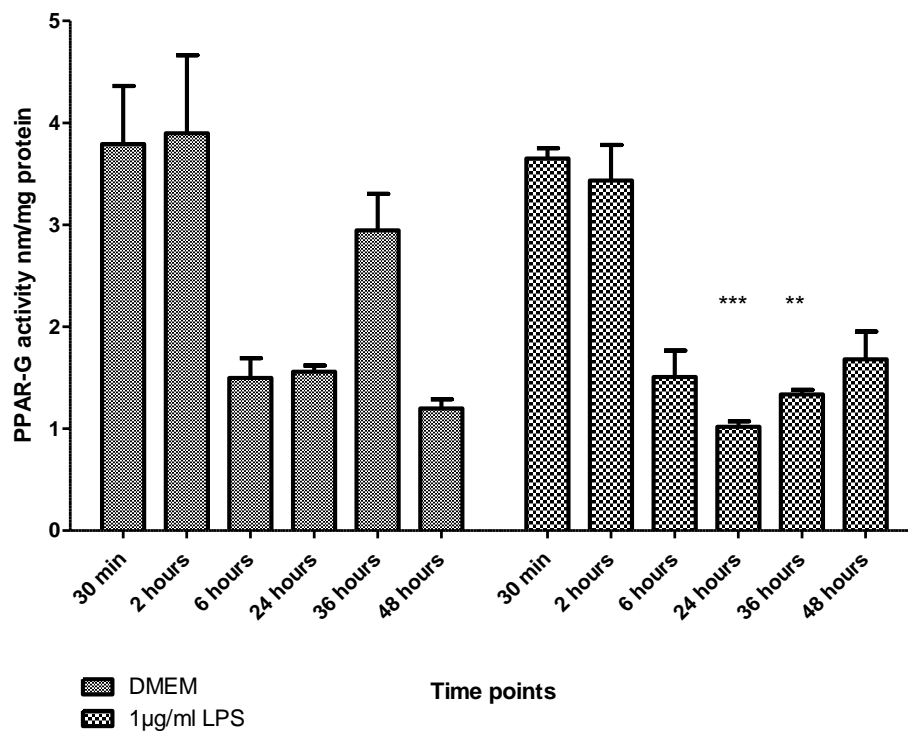


Figure 4.2.1: LPS reduced PPAR γ activity at 24 and 36 hours with no effect at other time points.

Cells were treated with LPS over 48 hours and the stimulations were terminated at different time points. Nuclear extractions were used to measure PPAR γ activity. The negative control was the unstimulated cells. Each bar represents the effect of LPS on PPAR γ activity.

The data shown were obtained from 3 independent experiments ($n=3$). One Way ANOVA with the Dunnett's post hoc test was carried out between the negative control and the samples where LPS was added to the cells, as represented by *. Data are expressed as mean \pm SEM (error bars); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

IL-4, as early as 30 minutes, significantly increased PPAR γ activity (8.22 ng/mg protein; $p < 0.01^{**}$) compared to the negative control (3.79 ng/mg protein) (fig 4.2.2a). Following this, IL-4 treated cells did not increase PPAR γ activity between 2 and 36 hours (5.78, 2.06, 2.13, 2.63 ng/mg protein) (fig 4.4.2). IL-4 significantly increased PPAR γ activity at 48 hours (20.62 ng/mg protein; $p < 0.01^{**}$) compared to the control (1.2 ng/mg protein). The addition of SB203580 to IL-4 treated cells showed a significant reduction in PPAR γ activity at all the time points investigated (0.06 ng/mg protein; $p < 0.001^{***}$, 0.06 ng/mg protein; $p < 0.001^{***}$, 0.06 ng/mg protein; $p < 0.001^{***}$, 0.06 ng/mg protein; $p < 0.001^{***}$, 0.07 ng/mg protein; $p < 0.001^{***}$, 0.05 ng/mg protein; $p < 0.001^{***}$) (fig 4.2.2b).

4.3 P38 MAPK involvement in LPS, diclofenac and IL-4 inductive COX-

2

P38 has been shown to stabilise COX-2 mRNA (Dean et al., 1999) and the addition of 0.1 μ M SB203580 was capable of inhibiting COX-2 mRNA in pro-inflammatory pathways induced by LPS. SB203580 is a compound that inhibits p38 by inhibiting activation of MAPKAPK. Despite previously being associated with pro-inflammatory signalling pathways (see for reviews: (Cuenda and Rousseau, 2007, Coulthard et al., 2009, Cuadrado and Nebreda, 2010)), studies have opened doors for an anti-inflammatory role for p38 (Kim et al., 2008, Guma et al., 2012, Jimenez-Garcia et al., 2015). Jimenez-Gracia *et al.*, (2015) showed p38 to play a crucial part in macrophage polarisation. This gave impetus to investigate the role of p38 in LPS, diclofenac and IL-4 induced COX-2. We have shown p38 to be activated following rosiglitazone treatment of cells in PPAR γ pathways leading to the induction of COX-2 (fig 4.1.1). Furthermore, we have shown p38 to mediate PPAR γ activity as, blocking p38 with 10 μ M SB203580 completely abolished PPAR γ activity (fig 4.2.2). To this end, whether inhibiting p38 using SB203580 has an effect on COX-2 expression and activity induced by various stimulants needs to be investigated.

Cells were plated to in T25 flasks, 24 hours before adding DMEM (negative control) or 1, 10 and 100 μ M SB203580 alone (negative controls). The positive controls were stimulations with 1 μ g/ml LPS, 500 μ M diclofenac or 10ng/ml IL-4. Following a 1-hour pre-treatment with DMEM, or 1, 10 and 100 μ M SB203580, DMEM or 1, 10 and 100 μ M SB203580 was added to the cells in the negative controls. In the experimental samples, 1, 10 and 100 μ M SB203580 was added with 1 μ g/ml LPS, 500 μ M diclofenac or 10ng/ml IL-4 for 24 and 48 hours. Post stimulations, cells were lysed with cell lysis

buffer and assayed for protein concentration (see 2.3). For Western blot analysis (see 2.4), 20µg protein was loaded on the gel and run at 100V for 1 hour. Anti-COX-2 and anti-β-Actin antibodies were added to PVDF membranes overnight. Secondary antibody was added for 1 hour before adding ECL to develop the membrane.

COX-2 expression induced by LPS at 24 hours was slightly reduced in the presence of 1µM and 10µM SB203580 (fig 4.3.1a). One hundred micromolar SB203580 reduced and inhibited LPS induced COX-2 expression at 24 and 48 hours respectively (fig 4.3.1a,b). Diclofenac induced COX-2 expression was reduced in the presence of the p38 inhibitor with near to complete inhibition with 100µM SB203580 (fig 4.3.1c). This is similar to IL-4 where, SB203580 caused a reduction in the intensity of COX-2 expression with concentrations as low as 10µM at 48 hours (fig 4.3.1d).

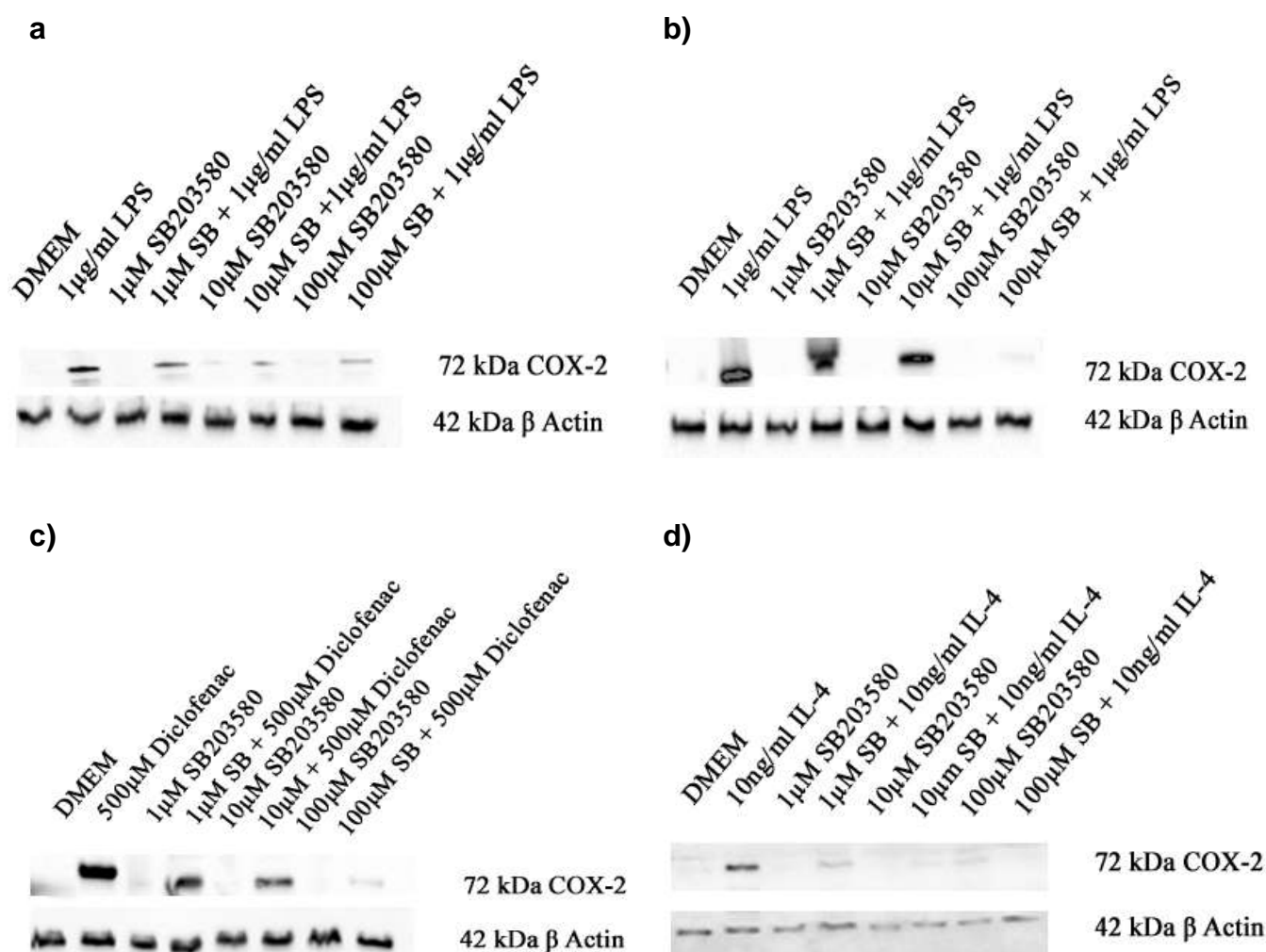


Figure 4.3.1 p38 inhibitor did not block LPS induced COX-2 expression at a) 24 hours. High concentrations of SB203580 inhibited COX-2 expression induced by b) LPS and c) diclofenac at 48 hours. Low concentrations of SB203580 inhibited d) IL-4 induced COX-2 expression at 48 hours.

Cells were treated with 1 μ g/ml LPS, 500 μ M diclofenac and 10 ng/ml IL-4 with and without increasing concentrations of SB203580 for 24 hours for LPS treatment and 48 hours for all treatments. At the end of the stimulations, cell lysates were analysed for both COX-2 and β -Actin. Unstimulated cells or cells treated with the p38 inhibitor alone were the negative controls. The positive controls were cells treated with LPS for 24 hours and LPS, diclofenac and IL-4 for 48 hours. All other bands represent the effect of the p38 inhibition on the COX-2 induced by the stimulants. The data shown were obtained from 3 independent experiments ($n=3$).

For ELISA experiments looking at PGE₂ levels, the optimal concentration of SB203580 was used (10 μ M). This was because 10 μ M SB203580 inhibited IL-4 induced COX-2 to a different intensity compared to the inhibition on LPS induced COX-

2 expression (fig 4.3.1). Furthermore, 10 μ M SB203580 was used by other groups to inhibit p38 MAPK (Huang et al., 2013, Jimenez-Garcia et al., 2015). This concentration was reported to inhibit IL-1 β induced COX-2. Cells were pre-treated with 10 μ M SB203580 for 1 hour before introducing the stimulant along with SB203580. At the end time points, the wells were washed with serum free media. Thirty micromolar arachidonic acid was added for 30 minutes before collecting the supernatant which was measured for COX-2 activity via ELISA techniques (see 2.5).

After examining COX-2 expression, the activity was measured with the working concentration of SB203580. The negative control, where cells were left unstimulated or treated with SB203580 showed no COX activity (5.92, 4.15 ng/mg protein respectively). At 24 hours, LPS induced COX-2 activity (87.04 ng/mg protein; $p < 0.001^{***}$). With the addition of the p38 inhibitor, the activity was significantly reduced (2.6 ng/mg protein; $p < 0.001^{$$$}$). At 24 hours, 500 μ M diclofenac did not induce COX-2 activity (15.95 ng/mg protein) and the addition of SB203580 had no effect on the activity of COX-2 (23.02 ng/mg protein) (fig 4.3.2). IL-4 did not induce COX-2 activity at 24 hours (3.99 ng/mg protein) and SB203580 did not affect this reading (9.52 ng/mg protein).

At 48 hours, the negative control and cells treated with SB203580 alone did not induce COX-2 activity (8.08, 5.97 ng/mg protein respectively). LPS did not induce COX-2 activity at 48 hours (22.85 ng/mg protein) however when the p38 inhibitor was added to the cells, the activity was significantly increased (35.11 ng/mg protein; $p < 0.01^{$$}$) (fig 4.3.2). This was in contrast to the diclofenac treated cells where diclofenac significantly induced COX-2 activity (48.94 ng/mg protein; $p < 0.05^{*}$) but the

addition of SB203580 significantly decreased this (21.36 ng/mg protein; $p<0.05^{\$}$). The same was true for 10ng/ml IL-4 at 48 hours, where IL-4 significantly induced COX-2 activity (57.79 ng/mg protein; $p<0.05^*$) but the addition of SB203580 significantly reduced this (23.77 ng/mg protein; $p<0.05^{\$}$) (fig 4.3.2).

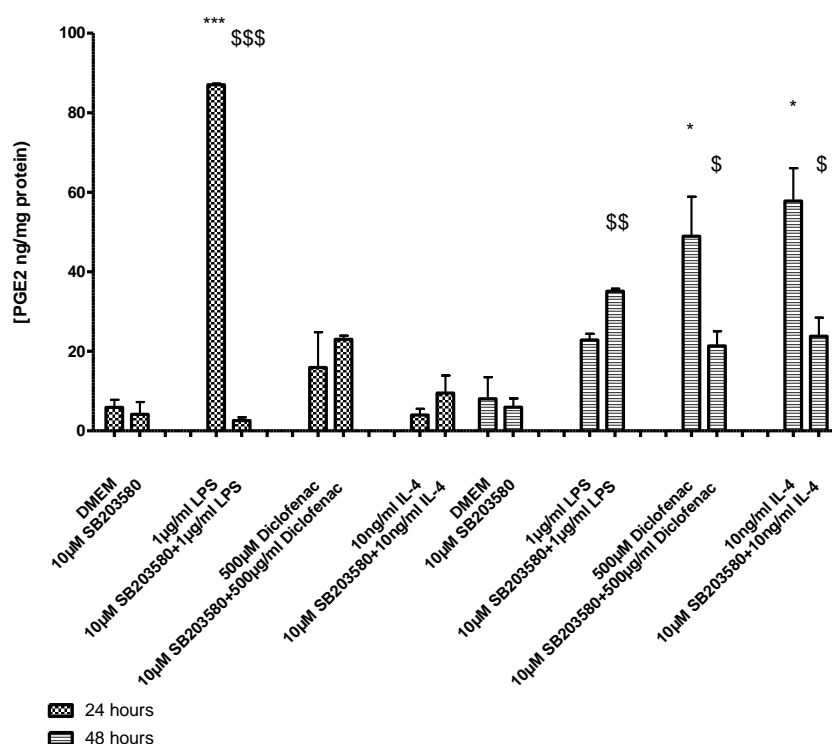


Figure 4.3.2: LPS required p38 to induce COX-2 activity at 24 hours. SB203580 increased LPS induced COX-2 activity but reduced diclofenac and IL-4 induced COX-2 activity at 48 hours.

Cells were treated with 1µg/ml LPS, 500µM diclofenac and 10ng/ml IL-4 for 24 or 48 hours in the absence and presence of 10µM SB203580. At the end of the stimulations, the supernatant was collected to measure COX-2 activity. The negative control were cells that were left unstimulated or treated with only SB203580. The positive control were cells treated with either LPS, diclofenac or IL-4. All other bars represent the effect of SB203580 on PGE₂ produced by LPS, diclofenac and IL-4.

The data shown were obtained from 3 independent experiments ($n=3$). One Way ANOVA with the Dunnett's post hoc test was used to compare the negative and positive controls, as represented by * and between the positive controls and samples where SB203580 was added, as represented by \$. Data are expressed as mean \pm SEM (error bars); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, \$ $p<0.05$, \$\$ $p<0.01$, \$\$\$ $p<0.001$.

4.4 Role of cAMP in the polarisation of macrophages into an M2 phenotype

cAMP is a second messenger, modulated by AC in a cascade of events within a complex signalling pathway (see for review: (Serezani et al., 2008, Yan et al., 2016)). cAMP has been associated with anti-inflammatory macrophages and the suppression of innate immune functions. It has been suggested that the immuno-stimulatory effects of mediators of immunity depend on intracellular cAMP thus, increased cAMP increases predisposition to infection (see for review: (Serezani et al., 2008)). It is widely accepted that M2 macrophages are involved in the pre-disposition to disease as suppression of pro-inflammatory responses restrict the host's ability to fight infection (see for review: (Cassetta et al., 2011)). cAMP and the downstream kinase, PKA have been targeted in the treatment of several diseases such as heart disease, due to their significance in such conditions (see for review: (Serezani et al., 2008, Yan et al., 2016)).

4.4.1 Requirement of cAMP in IL-4 induced COX-2

To understand the relevance of cAMP in the induction of COX-2 by IL-4, the cAMP antagonist, rp-cAMP was used (Bystrom et al., 2008). Downstream activation of COX-2 produce PGE₂ which binds the EP₄ receptor, thus augmenting the production of cAMP (Sokolowska et al., 2015). The question lies as to whether a positive feedback mechanism exists whereby the induction of COX-2 produces cAMP and whether this cAMP further induces COX-2.

Cells were plated in T25 flasks for 24 hours. Cells were treated with increasing concentrations of the cAMP antagonist, rp-cAMP, for 1 hour before stimulating with 1µg/ml LPS or 10ng/ml IL-4. Cells were treated for 48 hours and at the end time point, cell pellets were obtained (see 2.1). Treatment for 48 hours was used because IL-4 did not induce COX-2 at 24 hours. Cells were lysed and assayed for protein concentration (see 2.3) before looking for COX-2 expression (see 2.10).

Treatment of cells with 1µg/ml LPS for 24 hours showed COX-2 expression. With the addition of rp-cAMP, COX-2 bands were only slightly decreased but expression was obvious (fig 4.4.1.1a). rp-cAMP on its own, did not induce COX-2 expression. IL-4 induced the expression of COX-2 at 48 hours (fig 4.4.1.1b). In the presence of 5µM rp-cAMP, this expression was observed. With increasing concentrations of rp-cAMP (10-50µM), COX-2 expression induced by IL-4 was abolished completely (fig 4.4.1.1b).

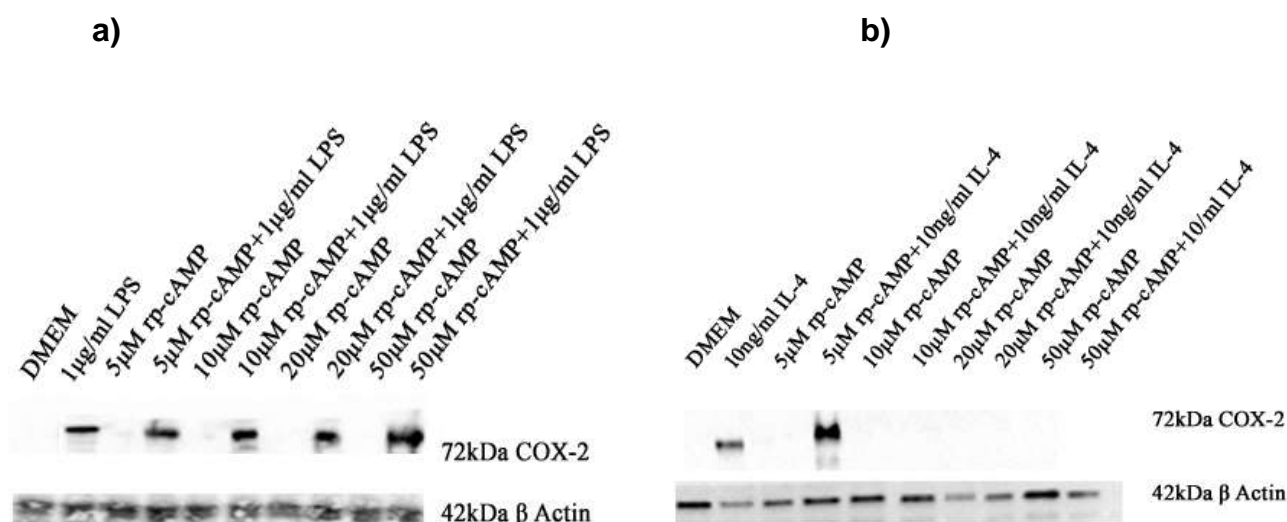


Figure 4.4.1.1: cAMP is not required for a) LPS induced COX-2 at 24 hours, but is critical for b) IL-4 induced COX-2 at 48 hours.

Cells were pre-treated with increasing concentrations of rp-cAMP for 1 hour before the addition of LPS for 24 hours or IL-4 for 48 hours. Cell pellets were collected and lysed before carrying out Western blot analysis. The expression of 72kDa COX-2 and 42kDa β -Actin was observed using the anti-COX-2 and anti- β -Actin antibodies. The negative control was the unstimulated cells and cells to which only rp-cAMP was added. The positive controls had no rp-cAMP added to them. The data shown were obtained from 3 independent experiments ($n=3$).

4.4.2 Role of p38 and PPAR γ in LPS and IL-4 induced cAMP

In order to understand what may modulate cAMP *in vitro*, experiments were carried out to antagonise PPAR γ with BADGE and inhibit p38 with SB203580. Interest in both PPAR γ and p38 was shed in work previously relating to a potentially anti-inflammatory COX-2 protein (Ayoub et al., 2009, Na et al., 2013, Jimenez-Garcia et al., 2015) as antagonism of PPAR γ (fig 4.1.2b), inhibition of p38 (fig 4.3.1) and antagonism of cAMP (fig 4.4.1.1b) blocked COX-2.

In a subset of experiments, the levels of cAMP produced by cells following stimulations was investigated. Cells were plated in a 96 well plate at 50×10^3 density 24 hours prior to stimulations. A preliminary experiment showed that this number of

cells was ideal to obtain a detectable cAMP concentration. Cells were treated with either 150 μ M of the PPAR γ antagonist, BADGE or 10 μ M of the p38 inhibitor, SB203580 for 1 hour. This was followed by either 24 or 48-hour stimulations with 1 μ g/ml LPS, 500 μ M diclofenac or 10ng/ml IL-4. cAMP production was measured using a cAMP assay kit. (Cell Signalling). The wells were washed and lysed using cell lysis buffer provided in the kit. The samples and standards were added to the wells along with HRP-linked cAMP solution. After 3 hours, the cells were washed and TMB substrate was added before the stop solution was added. Absorbance was measured at 450nm (see 2.6).

At 24 hours and 48 hours, 1 μ g/ml LPS did not induce cAMP production (0.33, 0.59 ng/mg protein respectively). The cAMP levels reported were basal as they were somewhat similar to the levels seen in the negative control at 24 and 48 hours (0.39, 0.33 ng/mg protein respectively). The addition of SB203580 to the LPS treated cells, caused a significant increase in cAMP production at both 24 and 48 hours (0.76 ng/mg protein; $p < 0.01^{\$}$, 0.94 ng/mg protein; $p < 0.05^{\$}$ respectively) (fig 4.4.2.1) compared to the LPS treated cells. At 24 hours, the addition of BADGE to the LPS treated cells, showed an increase in cAMP production (1.13 ng/mg protein; $p < 0.001^{$$$}$) compared to cells treated with LPS alone (0.33 ng/mg protein) (fig 4.2.1a). This effect was not seen at 48 hours, where BADGE had no effect on the LPS treated cells (0.65 ng/mg protein) (fig 4.4.2.1 b).

Diclofenac, at 24 and 48 hours, caused a significant increase in cAMP production (0.61 ng/mg protein; $p < 0.05^*$, 1.02 ng/mg protein; $p < 0.01^{**}$ ng/mg protein respectively). At 24 hours, the addition of SB203580 had no effect on diclofenac

induced changes to cAMP production (0.67 ng/mg protein) (fig 4.4.2.1a). At 48 hours, the surge in cAMP production by diclofenac was significantly reduced by SB203580 (0.29 ng/mg protein; $p<0.05^{\$}$) to levels similar to the negative control (0.33 ng/mg protein) (fig 4.4.2.1b). BADGE did not significantly reduce or increase levels of cAMP produced by diclofenac at either 24 or 48 hours (0.8, 0.61 ng/mg protein respectively) (fig 4.4.2.1). A non-significant reduction was observed at 48 hours in the presence of BADGE (0.61 ng/mg protein). The levels of cAMP, in the presence of BADGE, did not reach levels seen in the unstimulated cells at 24 and 48 hours (0.39, 0.33 ng/mg protein respectively) (fig 4.4.2.1).

IL-4 treated cells produced cAMP at 48 hours (0.85 ng/mg protein; $p<0.01^{**}$), but not 24 hours (0.48 ng/mg protein). cAMP levels, following IL-4 treatment at 24 hours, were similar to the negative control (0.39 ng/mg protein). The presence of the p38 inhibitor with IL-4 caused a significant increase in cAMP production at 24 hours (1 ng/mg protein; $p<0.01^{\$}$). At 48 hours, SB203580 caused a significant decrease in IL-4 induced cAMP production (0.23 ng/mg protein; $p<0.001^{\$ \$ \$}$) to levels, on average, lower than the negative control (0.33 ng/mg protein) (fig 4.4.2.1b). The addition of BADGE, similar to SB203580, caused a significant increase in cAMP production at 24 hours (0.99 ng/mg protein; $p<0.01^{\$}$) (fig 4.4.2.1a). At 48 hours, the level of cAMP was significantly reduced in the presence of BADGE and IL-4 (0.59 ng/mg protein; $p<0.01^{\$}$); however this level was, on average, greater than the amount of cAMP observed in the negative control (0.33 ng/mg protein) (fig 4.4.2.1b).

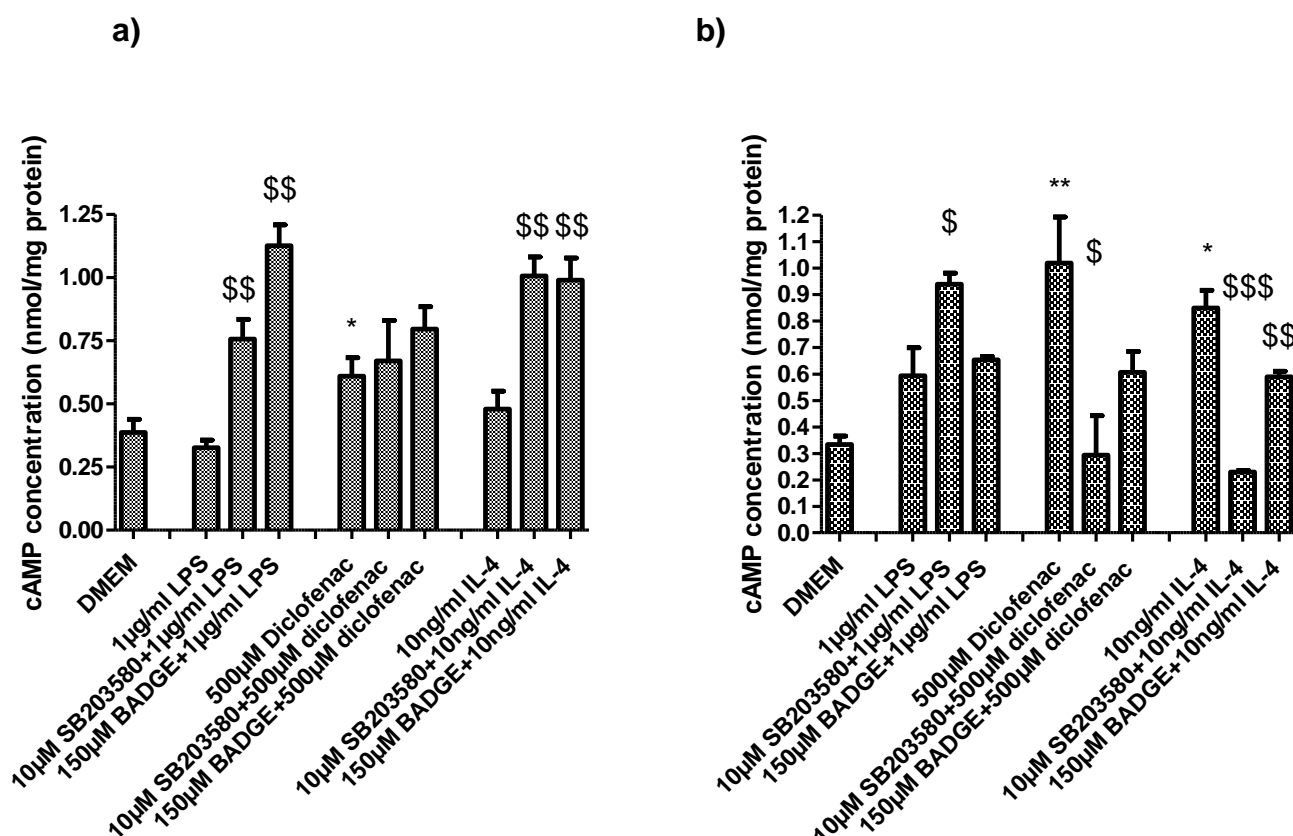


Figure 4.4.2.1: The effect of SB203580 and BADGE on cAMP levels following treatment of cells with 1µg/ml LPS, 500µM diclofenac and 10ng/ml IL-4 for a) 24 hours and b) 48 hours.

Cells were treated with 1µg/ml LPS, 500µM diclofenac and 10ng/ml IL-4 in the presence and absence of 10µM SB203580 or 150µM BADGE. After 24 and 48-hour stimulations, the cell lysate was analysed for cAMP production. The negative control was the untreated cells while the positive controls were the cells treated with either LPS, diclofenac or IL-4. All bars reflect the amount of cAMP present following the incubations.

The data shown were obtained from 3 independent experiments (n=3). T Test was carried out between the negative control and the positive control; as represented by *. T Test was also carried out between the positive control and the samples where either SB203580 or BADGE was used to pre-treat the cells. This is represented by \$. Data are expressed as mean+/- SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, \$p<0.05, \$\$p<0.01, \$\$\$p<0.001.

4.5 Discussion to results chapter 4

Signalling pathway involved in the induction of COX-2 by IL-4

4.5.1 Activation and requirement of PPAR γ in the IL-4 induced COX-2 pathway

As we have shown IL-4 to induce a catalytically active COX-2 protein (fig 7.4.1), we sought to investigate the signalling pathway involved in this induction. PPAR γ was given key importance previously (Ayoub et al., 2009) as a relevant transcription factor in the induction of COX-2 by diclofenac. Furthermore, it has been suggested that PPAR γ is anti-inflammatory and 15dPGJ₂, the natural ligand for this receptor, bares anti-inflammatory properties (Paintlia et al., 2006). This glitazone receptor was found to be activated in the induction of COX-2 when cells were treated with rosiglitazone, a known ligand for PPAR γ (fig 4.1.1). Increasing concentrations of rosiglitazone not only increased COX-2 induction at both 24 and 48 hours, but also activated p38 (fig 4.1.1).

To evaluate whether IL-4 increase PPAR γ activity in this cell line, initially cells were treated with 10ng/ml IL-4 for 24 and 48 hours. At the end of the stimulation, the samples were collected (see 2.1) and prepared for immunoblotting. The samples were run and anti-FABP4 antibody was used to measure FABP4; a PPAR γ target gene used as a measure of PPAR γ activity (Szanto et al., 2010). This protein is <20kDa and so a 15% gel was used. Despite loading 20 μ g protein, it remained difficult to probe this protein. As an alternative approach, using a semi-quantitative ELISA technique (see 2.7), we showed PPAR γ activation was completely absent in the presence of 1 μ g/ml LPS throughout the first 48 hours of stimulation (fig 4.2.1). In fact, LPS was found to

reduce PPAR γ expression both *in vivo* and *in vitro* (Simonin et al., 2002, Miksa et al., 2007, Necela et al., 2008, Zhou et al., 2008).

We observed 10ng/ml IL-4 to activate PPAR γ significantly at both 30 minutes and 48 hours (fig 4.2.2a). This directly links IL-4 to PPAR γ which is something that has previously been visited (Huang et al., 1999, Paintlia et al., 2006, Szanto et al., 2010). IL-4 was shown to augment PPAR γ expression in oligodendrocyte progenitor cells (Paintlia et al., 2006) and activity in macrophages and dendritic cells, as reflected by target gene expression (Szanto et al., 2010). Addition of the PPAR γ antagonist, GW9662, to glial cells, in the presence of IL-4 and a pro-inflammatory cytokine mix, showed IL-4 inductive effects to be lost, as shown by increased nitrite and iNOS expression (Paintlia et al., 2006). Furthermore, this group showed GW9662 to reverse the IL-4 effect on the survival of differentiating oligodendrocyte progenitors. Thus, there is evidence to support the notion that IL-4 activates PPAR γ and potentially exert its effects through this.

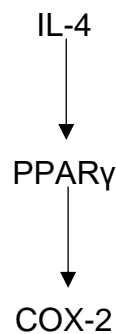
LPS treated cells have shown NF κ B to be activated and required in the induction of COX-2 (Kim et al., 2007, Ayoub et al., 2009). Whilst IL-4 activates PPAR γ , it was shown to inhibit NF κ B as shown by electrophoretic mobility shift assay (EMSA) data where, IL-4 reduced band intensity of p65 and p50, both NF κ B subunits (see 1.7). Upon addition of GW9662, this effect was reversed, thus suggesting PPAR activation may shut down NF κ B activation (Paintlia et al., 2006). This is largely ironic as both transcription factors are required for COX-2 induction. LPS did not activate PPAR γ (fig 4.2.1) and antagonising this did not impact LPS induced COX-2 expression in macrophage J774.2 cells (Ayoub et al., 2009) or activity (fig 4.1.3). Due to the work on

TLR/PPAR β/δ signalling pathways, interest developed in TLR/PPAR α/γ pathways in astrocytes. TLR4 and TLR1/2 agonists, LPS and peptidoglycan respectively, induce PGE₂ and TNF α in astrocytes. This was accompanied with reduced PPAR γ mRNA. This group found that blocking NF κ B with Bay 11-7085 reversed this effect, suggesting TLR to exert its effects via NF κ B (Chistyakov et al., 2015). Blocking TLR4 with 5 μ M CL1-095 removed LPS induced TNF α and LPS induced reduction in PPAR γ mRNA; suggesting the effect of LPS to be dependent on TLR4. The fact that IL-4 and LPS have different receptor activations downstream of binding its ligand, suggests two distinct pathways. Furthermore, there is strong evidence for NF κ B and PPAR γ to be on opposite ends of the spectre.

It is clear that IL-4 activates PPAR γ but in order to understand whether PPAR γ is actually required in the induction of COX-2, this receptor must be antagonised and protein expression must be determined. Similar to diclofenac induced COX-2, antagonising PPAR γ inhibited induction of COX-2 by IL-4 (fig 4.1.2). Furthermore, whereas PPAR γ inhibition by BADGE had no impact on LPS induced COX-2 activity, introduction of BADGE at 48 hours with both diclofenac and IL-4 inhibited COX-2 activity (fig 4.1.3). Thus, PPAR γ is both activated directly by IL-4 and is essential in the expression and catalytical activity of COX-2. Interestingly, IL-4 did not activate PPAR γ activity at 24 hours, (fig 4.2.2a) and COX-2 was not induced at this time point. In parallel, IL-4 activated PPAR γ at 48 hours (fig 4.2.2a) and COX-2 was expressed at this time (fig 3.2.1). This re-confirms the requirement for PPAR γ in the IL-4 pathway.

The conundrum here is, when PPAR γ activation can lead to COX-2 induction at 24 hours (fig 4.1.1) and PPAR γ is activated by IL-4 as early as 30 minutes (fig

4.2.2a), why does IL-4 induce COX-2 as late as 48 hours only. We assume there is a translational delay for the COX-2 protein which controls the time of expression.



4.5.2 Involvement of P38 MAPK in COX-2 induction

To this end, we developed interest in the kinase, p38. Initially, it was noted whether p38 was required for the induction of COX-2 as we predict p38 activation upstream to COX-2 translation. Thus, cells were treated with the p38 α/β inhibitor SB203580, in the presence of the stimulants and assessed for COX-2 expression. This drug was previously found to inhibit COX-2 mRNA at a transcriptional level (Dean et al., 1999). p38 has been reflected as a stabiliser of COX-2. Inhibition of p38 with low concentrations of SB203580 showed sustained LPS induced COX-2 expression at 48 hours (fig 4.3.1a, b). Only high concentrations of SB203580 (100 μ M) blocked LPS induced COX-2. Diclofenac induced COX-2 expression was merely reduced by the addition of the pyridinyl imidazole inhibitor, SB203580, (fig 4.3.1c), however in IL-4 treated cells, COX-2 expression was lost (fig 4.3.1d). This suggests p38 may not always be required for the induction of COX-2; however, it is crucial in the IL-4 pathway.

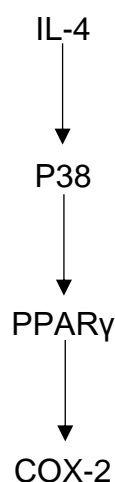
The optimal working concentration from this experiment was 10 μ M SB203580 because it specifically inhibited IL-4 induced COX-2 but not LPS. This concentration

was also used by Jimenez-Garcia et al. (2015) to look at the effect of SB203580 on M2 markers. We were able to show that the COX-2 activity induced by LPS was significantly reduced in the presence of 10 μ M SB203580 (fig 4.3.2). This was expected based on the immense number of publications that looked at LPS induced p38 regulation of mRNA. We were able to show COX-2 activity being regulated by p38, upon stimulation with IL-4 and diclofenac, as blocking p38 significantly reduced COX-2 activity (fig 4.3.2). This differed to COX-2 activity measured upon treatment of cells with LPS and SB203580 together, where, p38 blockage led to increased COX-2 activity at 48 hours. This correlates well with the protein expression data (fig 4.3.1).

We were able to show p38 as a key mediator of PPAR γ activity induced by IL-4 (fig 4.2.2b). Whereas IL-4 could induce activity of PPAR γ as early as 30 minutes (fig 4.2.2a), all PPAR γ activity was lost when p38 was inhibited by introducing SB203580 (fig 4.4.2b). This suggests p38 activation upstream to PPAR γ activation, but downstream to IL-4 treatment of cells; something which was previously observed (Schild et al., 2006). Interestingly, Yano et al. (2007) reported inhibition of PPAR γ induction following treatment with p38 inhibitor. This suggests an intricate relationship between PPAR γ and this MAPK. This group found PPAR γ not to impact p38 however p38 inhibition attenuated PPAR γ protein stability in primary trophoblasts. In astrocytes, SB203580 was shown to modulate the rate of PPAR degradation and reduce PPAR γ activity (Chistyakov et al., 2015). This supports our finding of p38 regulation of PPAR γ .

P38 inhibitors are currently popular in the treatment of chronic inflammation due to the acknowledged pro-inflammatory properties of p38 (see for review: (Kumar et al., 2003)). However, p38 is increasingly being recognised to play a dual role in

inflammation (Jimenez-garcia *et al.*, 2015). IL-4 leading to the activation of the anti-inflammatory receptor, PPAR γ (fig 4.2.2a) suggests an anti-inflammatory pathway downstream of ligand binding. One of the end products of this being COX-2 activation, suggests a potential anti-inflammatory role for COX-2. As p38 is required for the proposed anti-inflammatory COX-2, this suggests that p38 inhibitors may be harmful depending on the time of administration. Findings by Jimenez-Garcia *et al.* (2015) show pharmacological inhibition and silencing of p38 to block expression of the M2 markers: Arg-1, Ym-1 and Fizz1. Furthermore, this group showed inhibition of phospho-STAT6 expression in the presence of p38 inhibitors. Guma *et al.* (2012) showed deletion of p38 α to increase subacute inflammatory arthritis with increased inflammatory cell infiltrate. Furthermore, inhibiting p38 α/β reduced IL-10 expression in both BMDM and RA synovium. Thus, these groups found p38 deficiency to increase the severity of inflammation (Guma *et al.*, 2012) and give rise to a pro-inflammatory environment and pro-inflammatory macrophages. Ananieva *et al.* (2008) like Kim *et al.* (2008) showed MSK, which is activated downstream to p38, as key in p38 α induced anti-inflammatory pathways.



4.5.3 Role of cAMP in the induction of COX-2 by IL-4

cAMP is an essential second messenger endowed with functions in modulating inflammatory processes; essentially through PKA activation. cAMP agonists have been found to resolve neutrophilic inflammation (Lima et al., 2017). Ligands which lead to AC activation and PDE mediated degradation control the balance of intracellular cAMP. PDE4 is believed to be the predominant isoenzyme in inflammatory cells and plays a crucial role in inflammation (Page and Spina, 2011).

To study the significance of cAMP in COX-2 induction, whether its activation is triggered during various stimulations and whether cAMP is relevant for the induction of COX-2 protein expression was investigated. Previous research has given evidence for the importance of cAMP response element in the expression of COX-2 (see for review: (Klein et al., 2007)). It has previously been documented that activation of cAMP results in increased COX-2 mRNA and protein expression however the amount varies for each cell line (see for review: (Klein et al., 2007)). In human myometrial cells, cAMP agonists were shown to increase COX-2 mRNA and protein and PG production (Chen et al., 2012). PGE₂ drives M2 polarisation as measured by expression of MR and IL-10 (Montero et al., 2016).

As we hypothesise the phenotype of the macrophage to dictate the temporal expression of COX-2, we looked into the relevance of cAMP in macrophages treated with both a pro-inflammatory and anti-inflammatory stimulus. The significance of cAMP in macrophage polarisation was previously visited by (Bystrom et al., 2008). This group found that M1 macrophage production of TNF α was disrupted and IL-10 production was elevated by db-cAMP, the cAMP analog. This suggests a reversion from an M1

macrophage into an M2 macrophage (Bystrom et al., 2008). cAMP agonists were found to increase the expression of AnxA1, an endogenous element that mediates the pro-resolving effects of cAMP (Lima et al., 2017). Neutralising this with anti-AnxA1 serum stopped resolution and the effects of db-cAMP were reversed. This suggests the effect of cAMP to occur via PKA and AnxA1; whose N-terminus is associated with its anti-inflammatory properties (Lima et al., 2017).

It appears that cAMP plays a role in the IL-4, but not the LPS, induced COX-2 pathway. Blocking cAMP with rp-cAMP, the cAMP antagonist, blocked expression of IL-4 induced COX-2 completely at high concentrations (fig 4.4.1.1b); with no such effect on LPS induced COX-2 (fig 4.4.1.1a). This immediately reflects a role of cAMP in the pathway by which IL-4 induces COX-2. The fact that COX-2 has differential requirements for the induction depending on the stimulant implies the existent of two different induction pathways.

PGE₂ has been identified as a ligand that activates AC to elevate cAMP levels. Thus, there appears to be a positive feedback mechanism, where cAMP induces COX-2 and the PG product drives cAMP release. The effect of PGE₂ is a complicated virtue as it has both immuno-stimulatory and immuno-suppressive properties (see for review: (Willoughby et al., 2000)). It is clear that cAMP production occurs upstream to COX-2 induction. Previous work has shown the COX-2 product, PGE₂, to stimulate cAMP production (Sokolowska et al., 2015) (see 1.8.2). A positive feedback mechanism may exist whereby cAMP stimulates COX-2 induction and this in turn maintains high levels of cAMP in the presence of the COX-2 product, PGE₂. The natural next step would be

to see whether either LPS, diclofenac or IL-4 were capable of inducing cAMP production and the role of PPAR γ and p38 on this.

Although cAMP was not significantly elevated in the presence of 1 μ g/ml LPS at both 24 and 48 hours, introducing the p38 inhibitor SB203580, significantly increased this at both time points (fig 4.4.2.1). Previously, p38 was shown to be activated as early as 10 minutes followed by activation of PKA and COX-2 induction (Chen et al., 1999). This supports our observations of p38 activation being upstream to cAMP, thus a potential regulatory function on cAMP regulation. BADGE had a similar effect on cAMP production following treatment of cells with LPS at 24 hours (fig 4.4.2.1b). Although, cAMP is commonly released in the early stages of any signalling pathway, a continuous positive feedback loop may keep cAMP production constant. These findings lead to the assumption that p38 and PPAR γ delay cAMP production. From this, it can also be assumed that both p38 and PPAR γ have some form of regulatory effect on AC, as this is what produces cAMP. Interestingly, diclofenac treatment on macrophages produce cAMP at both 24 and 48 hours. Neither p38 (fig 4.4.2.1c) nor PPAR γ (fig 4.4.2.1d) had any effect on this, except at 48 hours, where blocking p38 significantly reduced cAMP production (fig 4.4.2.1c). The fact that LPS did not produce cAMP but diclofenac did, leads us to believe the LPS treated macrophage may be pro-inflammatory and the diclofenac treated macrophage may be anti-inflammatory. This agrees with the cytokine profile post-diclofenac treatment in macrophages (Ayoub et al., 2009).

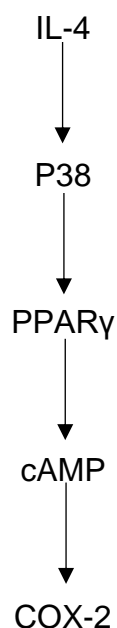
Collating data from these two experiments, we can conclude that cAMP may not be relevant in the LPS induced pathway leading to COX-2 expression. Interestingly, db-cAMP has previously been shown to possess opposing functions to LPS. db-Camp, being a cAMP agonist, was shown to increase cAMP after 1 hour. In a LPS induced self-resolving model of inflammation, there is an influx of neutrophils into the pleural cavity. Treatment with db-cAMP significantly reduced this number while increasing AnxA1 expression when introduced at the peak inflammatory time (Lima et al., 2017). Thus, LPS and cAMP elevating agents such as db-cAMP, have opposing roles in an inflammatory cell. This may explain why rp-cAMP had no notable effect on LPS induced COX-2 expression (4.4.1.1) in what we regard as the pro-inflammatory pathway. Contrary to this, whereas LPS could induce COX-2 by 3 hours, this stimulus was found to induce cAMP by 6 and 24 hours (Chen et al., 1999); suggesting COX-2 to precede cAMP production. Thus, Chen et al. (1999) suggested COX-2 induced PGE₂ to behave as an autocrine mediator for cAMP production.

It may well be that the effect of LPS on cAMP differs per cell line. Whereas low concentrations of LPS did not induce IL-33 production, a cAMP analog caused an increase in this; thus, and it was suggested that cAMP enhance the effect of LPS. This effect was inhibited by H89, a PKA inhibitor; thus highlighting the relevance of PKA in the LPS pathway in RAW264.7 macrophages (Sato et al., 2016). Chen et al. (1999) used the nitrite assay to show that inhibition of PKA reduced LPS induced iNOS expression whereas PKA activators increased iNOS expression. Raddassi et al. (1993) showed that in murine peritoneal macrophages, LPS induced cAMP and this second messenger was important in the retro-inhibitory control of the LPS induced PG production. One micromolar PGE₂ and LPS administration decreased NO synthase

whereas 1 μ M indomethacin suppressed PG production while increasing NO synthase (Raddassi et al., 1993).

Of high interest, IL-4 did not stimulate cAMP production in macrophage J774.2 cells at 24 hours however it was increased by 48 hours. We have shown IL-4 to induce COX-2 at this time point (fig 3.2.1). This cAMP may be induced via production of PGE₂ by COX-2 (fig 3.2.2) which may bind EP₄ receptors. This is somewhat ambiguous as, addition of the cAMP antagonist previously blocked IL-4 induced COX-2 expression (fig 4.4.1.1b). This implies cAMP production to occur before COX-2 induction and a positive feedback loop may sustain cAMP production. If IL-4 does induce cAMP before the 48-hour mark, this may be blocked by other mediators as it was not detectable at 24 hours. In the presence of SB203580 (fig 4.4.2.1e) and BADGE (fig 4.4.2.1f), at 24 hours, there was a significant increase in cAMP. At 48 hours, this effect was reversed and both SB203580 (fig 4.4.2.1e) and BADGE (fig 4.4.2.1f) caused significant reduction in cAMP levels. In correlation with this, we have shown BADGE and SB203580 to block COX-2 expression and PGE₂ production at 48 hours. Correlating these 2 pieces of evidences, it may be that cAMP is required for the translation of COX-2 protein and PG production. Chang et al. (2000) recorded increased intracellular cAMP and p38 activation prior to increased arginase activity; an indicator of macrophage polarisation. This goes hand in hand with the literature which provides strong evidence for cAMP to be involved in the M2 macrophage phenotypic state (Bystrom et al., 2008); where arginase activity is increased as determined by the concentration of urea produced. Arginase activity was found to be inhibited by SB203580, thus suggesting a requirement of p38 in urea production.

It would be interesting to observe whether IL-4 can stimulates cAMP at 30 minutes, where IL-4 first activates PPAR γ (fig 4.2.2a). It is possible that PPAR γ activation leads to downstream activation of a mediator which mediates COX-2 expression. This may be cAMP, because, blocking cAMP blocks COX-2 expression by IL-4 (fig 4.4.1.1) and PPAR γ was found to mediate cAMP production (fig 4.4.2.1). This also suggests that the activation of PPAR γ and the release of cAMP is somewhat immediate as this occurs within the same time point. Singh et al. (2015) previously suggested relaxin to induce cAMP upstream to PPAR γ activation via p38. Furthermore, Gabrielli et al. (2014) found that 3-isobutyl-1-methylxanthine, an inhibitor of PDE, increases cAMP and PKA activation on top of PPAR γ protein expression (Gabrielli et al., 2014); thus, pointing to a relation between cAMP and PPAR γ . It may be that, there is a positive feedback mechanism whereby, cAMP production is instigated by the upstream PPAR γ /p38; and the cAMP continues to induce activation of this pathway.



4.6 Chapter conclusion

This chapter gives strong evidence for the involvement of cAMP, PPAR γ and p38 in the induction of COX-2 by IL-4. Comparative analyses show differential requirements for these mediators in COX-2 induction by LPS and IL-4. Whereas IL-4 activates cAMP (fig 4.4.2.1) and requires cAMP (fig 4.4.1.1b) and PPAR γ (fig 4.1.2b, 4.1.3) for COX-2 induction, LPS did not. Accordingly, we suggest the IL-4 to bind its cognate receptor and activate p38 which mediates PPAR γ activity (fig 4.2.2). Furthermore, IL-4 induced COX-2 with a temporal delay in comparison to LPS induced COX-2 (fig 3.2.1). We suggest this delay to be mediated at the level of p38 and PPAR γ . PPAR γ activity produced cAMP which assists in the induction of COX-2. Blocking these mediators blocked the expression of COX-2 protein, confirming our speculative induction pathway. Following confirmation of the IL-4 induced COX-2 pathway, the phenotypic nature of the macrophage should be understood. As we hypothesise that the macrophage phenotype depicts the functional properties of COX-2, the phenotype post-stimulation needs to be evaluated.

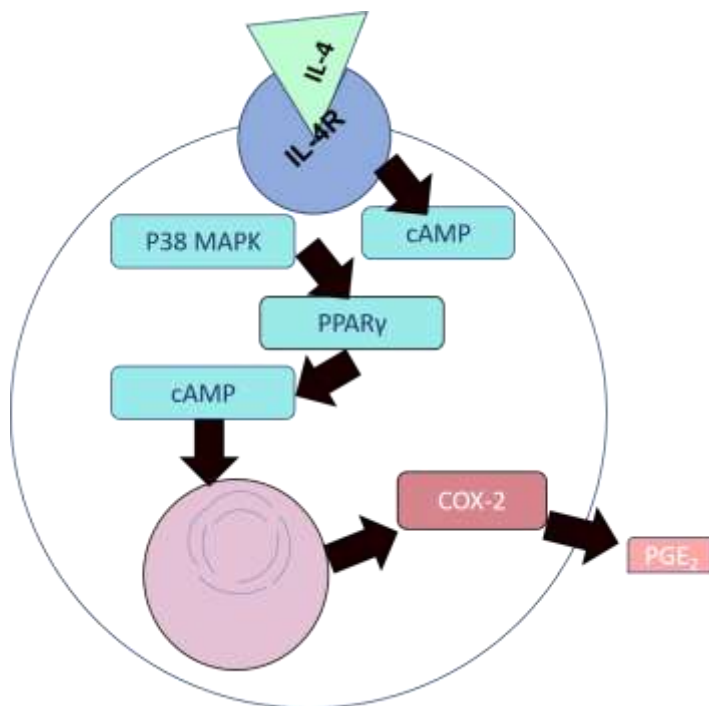


Figure 4.6.1 Diagram to conclude chapter

Macrophages treated with 10ng/ml IL-4 for 48 hours, produced cAMP, a common second messenger that has roles in anti-inflammatory signalling pathways. Inhibition of cAMP with *rp*-cAMP blocked COX-2 expression by IL-4. Furthermore, inhibition of both PPAR γ activity and p38 by BADGE and SB203580 respectively, blocked COX-2 activity. This confirmed PPAR γ , p38 and cAMP to lie upstream to COX-2 activation and are key in the induction pathway.

Chapter 5: Phenotype of the macrophage expressing COX-2 following stimulations with LPS and IL-4

5.1.1 Macrophage polarisation

There is a general conception that M2 macrophages typically produce high levels of IL-10 and low levels of TNF α while M1 macrophages produce low levels of IL-10 and high levels of TNF α . During infection, macrophages are a major source of IL-10 (see for review: (Couper et al., 2008)). IL-10 is an anti-inflammatory cytokine that inhibits pro-inflammatory responses; thus, IL-10 facilitates clearance of pathogens whilst alleviating immunopathology. IL-10 inhibits major histocompatibility complex (MHC) class II, thereby limiting pro-inflammatory cytokine production. It has been suggested that IL-10 can prevent neighbouring macrophages from polarising towards a pro-inflammatory phenotype (see for review: (Couper et al., 2008)). TNF α on the other hand polarises macrophages towards a pro-inflammatory phenotype and induces pro-inflammatory responses such as leukocyte adhesion (see for review: (Bradley, 2008)). Blocking TNF α has been shown to treat inflammatory conditions including RA (see for review: (Bradley, 2008)). cAMP, a key molecule in anti-inflammatory macrophages, has been shown to influence these two cytokines which are key as an indication of macrophage phenotype (Shames et al., 2001, Bystrom et al., 2008, Ayoub et al., 2009).

It has previously been observed that LPS treated macrophages produce TNF α (Parameswaran and Patial, 2010). IL-4 has been shown to polarise macrophages to an M2 phenotype and activate anti-inflammatory transcription factors and markers (Kaplan et al., 1996, Levings and Schrader, 1999, Szanto et al., 2010, Sheldon et al.,

2013). It is important to note whether the IL-4 treated cells, which were shown to express COX-2, exhibit anti-inflammatory or pro-inflammatory properties.

5.1.2 Cytokine profiling as a measure of macrophage phenotype state

Using cytoplasmic dot hybridisation mRNA analysis, it was previously suggested that the secretion patterns of lymphokines were definitive of the type of T helper cell present (Cherwinski et al., 1987). These cytokines could activate B cells differentially and mediate the regulatory functions downstream. Th1 cells produced IFN γ while Th2 cells produced IL-4 (Cherwinski et al., 1987). Thus, in a similar approach, we set out to measure the release of cytokines to understand the phenotype of the macrophage. To elucidate the phenotypic state of the macrophages following treatment of cells with LPS or IL-4, analysing the cytokine profile would be informative. ELISA (R&D Systems) was used to measure the anti-inflammatory cytokine, IL-10 and the pro-inflammatory cytokine, TNF α . TNF α mediates innate immunity and is implicated in inflammatory disease pathogenesis (see for review: (Bradley, 2008, Sabio and Davis, 2014, Kalliolias and Ivashkiv, 2016)). IL-10 is related to both adaptive and innate immunity with anti-inflammatory properties (see for review: (Bradley, 2008)). These cytokines were previously measured by Ayoub et al. (2009).

Of interest, whether a cell can polarise from one state to another *in vitro* has been questioned. Bystrom et al. (2008) previously showed the presence of the cAMP agonist, db-cAMP, to increase IL-10 whilst reducing TNF α in what was regarded as an M1 macrophage converting to an M2 phenotype; suggesting macrophages can revert from one phenotype to the other based on the surrounding milieu. This was also observed by Ghosh et al. (2016) who suggested the microenvironment to dictate

macrophage phenotype as injection of IL-4 into an M1 dominated spinal cord, allowed macrophages to polarise into an M2 phenotype. This puts great emphasis on the microenvironment surrounding the cells.

5.2 Phenotypic state of the macrophage following IL-4 treatment

Cells were plated in T25 flasks and stimulated with increasing concentrations of IL-4 for 2, 24 and 48 hours. Two hours was selected as we wanted to see whether IL-4 was capable of inducing this change in phenotype at an earlier stage; suggesting macrophage polarisation to take place before COX-2 protein expression. The supernatant was collected and analysed for IL-10 levels via ELISA (for protocol see 2.8).

All concentrations of IL-4 caused a significant increase in the levels of IL-10 produced from as early as 2 hours (7.95 ng/mg protein; $p < 0.01^{**}$ (5ng/ml IL-4), 20.55 ng/mg protein; $p < 0.01^{**}$ (10ng/ml IL-4), 14.6 ng/mg protein; $p < 0.01^{**}$ (25ng/ml IL-4), 15.36 ng/mg protein; $p < 0.01^{**}$ (50ng/ml IL-4), 11.34 ng/mg protein; $p < 0.01^{**}$ (100ng/ml IL-4)) (fig 5.2.1). At 24 hours, IL-10 levels were significantly higher (18.59 ng/mg protein; $p < 0.01^{**}$ (5ng/ml IL-4), 23.14 ng/mg protein; $p < 0.01^{**}$ (10ng/ml IL-4), 19.97 ng/mg protein; $p < 0.01^{**}$ (25ng/ml IL-4), 16.89 ng/mg protein; $p < 0.01^{**}$ (50ng/ml IL-4), 14.89 ng/mg protein; $p < 0.01^{**}$ (100ng/ml IL-4)) compared to the control (undetectable IL-10 levels) (fig 5.2.1). The same was true at 48 hours (14.24 ng/mg protein; $p < 0.05^{*}$ (5ng/ml IL-4), 19.66 ng/mg protein; $p < 0.01^{**}$ (10ng/ml IL-4), 19.56 ng/mg protein; $p < 0.01^{**}$ (25ng/ml IL-4), 18.28 ng/mg protein; $p < 0.01^{**}$ (50ng/ml IL-4), 20.76 ng/mg protein; $p < 0.01^{**}$ (100ng/ml IL-4)) (fig 5.2.1). The production of IL-10 was not concentration dependent. The levels of IL-10 produced, seem to be fairly

consistent with a small spread across the 3 time points investigated. The negative control showed undetectable IL-10 production at all the time points studied.

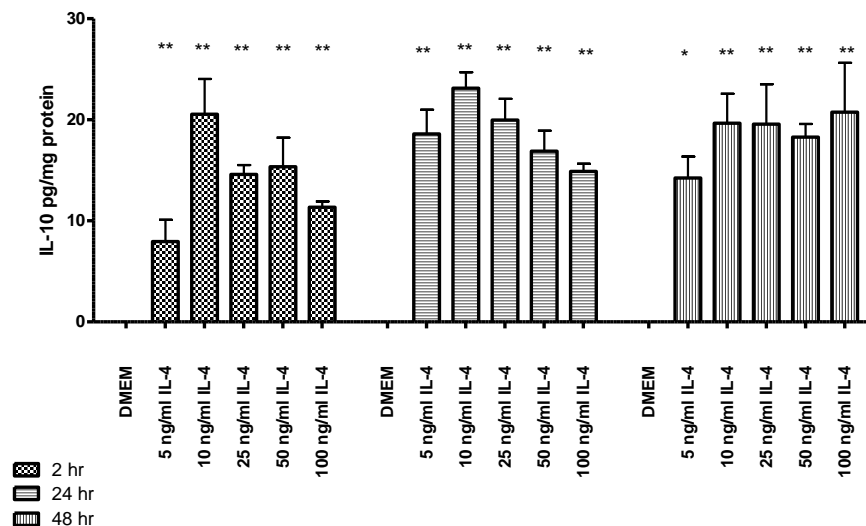


Figure 5.2.1: Increasing concentrations of IL-4 caused an increase in IL-10 levels between 2 and 48 hours.

Cells were treated with increasing concentrations of IL-4 ranging from 5ng/ml to 100ng/ml for 2, 24 or 48 hours. The supernatant was collected to analyse IL-10 levels. The negative control was cells that were left untreated. All other bars reflect the levels of IL-10 following treatment of cells with IL-4.

The data shown were obtained from 3 independent experiments (n=3). One Way ANOVA was carried out between the negative control and samples where increasing concentrations of IL-4 were added; represented by *. Data are expressed as mean+/- SEM (error bars); *p<0.05, **p<0.01, ***p<0.001.

5.3 Effect of p38 inhibition and PPAR γ antagonism on IL-4 induced production of IL-10

The relevance of p38 in IL-4 signalling and M2 macrophage polarisation has been visited beforehand (Jimenez-Garcia et al., 2015). Similarly, the relevance of PPAR γ in IL-4 signalling has also been documented (Szanto et al., 2010). Both SB203580 and BADGE have been shown to impact cAMP levels (fig 4.1.2), a molecule related to anti-inflammatory macrophages. As we have previously shown

ways in which both PPAR γ and p38 regulate COX-2 induction, with the aim of drawing parallelism between macrophage phenotype and COX-2 induction, the effect of both of these on cytokine production should be clarified. To this end, in a subset of experiments, the role of both p38 and PPAR γ in the production of IL-10 was investigated. Cells were pre-treated with BADGE and SB203580 for 1 hour, prior to the addition of 10ng/ml IL-4 for both 24 and 48 hours. At the end of these time points, the supernatant was collected and assayed for IL-10 levels via ELISA (see 2.8).

Upon addition of 10ng/ml IL-4 there was a significant upsurge in IL-10 at 24 hours (102 ng/mg protein; $p < 0.01^{**}$) compared to the unstimulated cells (undetectable IL-10 levels). This was significantly reduced by the addition of the p38 inhibitor (4.65 ng/mg protein; $p < 0.01^{**}$) (fig 5.3.1a). The negative control and cells treated with SB203580 alone, showed undetectable IL-10 levels. At 48 hours there was a significant increase in IL-10 when cells were treated with 10ng/ml IL-4 (76.68 ng/mg protein; $p < 0.001^{***}$). The addition of the p38 inhibitor completely blocked IL-10 production (undetectable IL-10 levels; $p < 0.001^{***}$) (fig 5.3.1a). The addition of BADGE to cells treated with 10ng/ml IL-4 significantly inhibited IL-10 production (undetectable IL-10 levels; $p < 0.001^{***}$ ng/mg protein).

In LPS treated cells, IL-10 was not produced. This level was not effected by the addition of either the p38 inhibitor or PPAR γ antagonist (data not shown). The LPS treated cells therefore showed the same amount of IL-10 as the unstimulated cells.

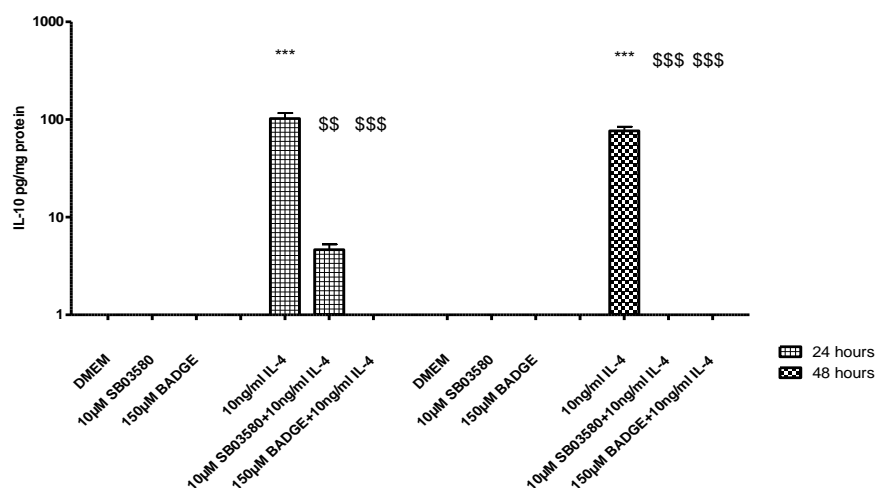


Figure 5.3.1 IL-4 induced IL-10 levels was significantly reduced at both 24 and 48 hours in the presence of SB203580 and BADGE.

Cells were treated with SB203580 or BADGE for 1 hour before adding 10ng/ml IL-4 to the cells for 24 and 48-hours. The supernatant was collected and analysed for IL-10 production. All readings were standardised to the respective protein concentration. Unstimulated cells and cells treated with SB203580 or BADGE were the negative controls. Cells treated with 10ng/ml IL-4 alone was the positive control. All bars represent the effect of either SB203580 or BADGE on IL-10 production by IL-4.

The data shown were obtained from 3 independent experiments ($n=3$). IL-10 levels produced following treatment of cells with IL-4 were statistically compared to the negative control via the One Way ANOVA; as represented by *. The positive control was compared to the samples that were co-treated with IL-4 and BADGE or SB203580 via the One Way ANOVA; represented by \$. Data are expressed as mean \pm SEM (error bars); * $p<0.05$, ** $p<0.01$, *** $p<0.001$, \$ $p<0.05$, \$\$ $p<0.01$, \$\$\$ $p<0.001$.

5.4 Effect of SB203580 and BADGE on IL-4 and LPS induced TNF α levels

Treating cells with 10ng/ml IL-4 for 24 hours had no real effect on TNF α levels (3.4 ng/mg protein) when compared to the unstimulated cells (2.51 ng/mg protein). This value was left unaffected when the cells were pre-treated with SB203580 (5.1 ng/mg protein) (fig 5.4.1a). The levels reported here were similar to that observed in the negative control (2.51 ng/mg protein) and in cells treated with only SB203580 (4.1

ng/mg protein). The same was seen at 48 hours where, upon co-treating cells with 10ng/ml IL-4 and 10 μ M SB203580, no change in TNF α was noted (4.67 ng/mg protein); similar to when cells were treated with IL-4 alone (3.98 ng/mg protein). Adding SB203580 to IL-4 treated cells, therefore, had no effect on TNF α production. From this it can be assumed that IL-4 did not activate any transcription factor leading to TNF α synthesis. This may be because the IL-4 induced an anti-inflammatory environment.

Co-treating macrophages with 10ng/ml IL-4 and 150 μ M BADGE showed a significant increase in the production of TNF α at 24 hours (7.24 ng/mg protein $p<0.05^{\$}$) (fig 5.4.1b). BADGE on its own had no effect on TNF α levels (4.07 ng/mg protein) compared to the negative control (2.51 ng/mg protein). At 48 hours, levels of TNF α were not significantly effected by the addition of BADGE (3.19 ng/mg protein) (fig 5.4.1b). Despite this, when BADGE was added to the cells without a stimulant, there was a significant reduction in the TNF α produced at 48 hours (2.03 ng/mg protein $p<0.05^{\#}$).

At 24 hours, upon addition of 1 μ g/ml LPS, there was a significant increase in TNF α production (1274.59 ng/mg protein; $p<0.001^{***}$). This was significantly reduced when SB203580 was added (251.34 ng/mg protein; $p<0.001^{\$ \$ \$}$); however levels were still higher than the negative control (2.51 ng/mg protein) (fig 5.4.1c). At 48 hours, the negative control showed basal levels of TNF α (5.09 ng/mg protein), slightly higher than what was observed at 24 hours (fig 5.4.1c). Treatment of cells with 10 μ M SB203580 for 48 hours showed low levels of TNF α production (8.76 ng/mg protein), similar to the unstimulated cells. LPS stimulation of macrophages for 48 hours did not significantly

increase TNF α levels (37.59 ng/mg protein). The addition of SB203580 did not impact TNF α levels (14.74 ng/mg protein) (fig 5.4.1c).

At 24 hours, co-treatment of 150 μ M BADGE and 1 μ g/ml LPS showed a significant decrease in TNF α levels (4.42 ng/mg protein; $p < 0.001^{$$$}$) compared to cells treated with just 1 μ g/ml LPS (1274.59 ng/mg protein; $p < 0.001^{***}$) (fig 5.4.1d). At 48 hours, the addition of BADGE significantly reduced TNF α levels (2.03 ng/mg protein; $p < 0.05^{\#}$) (fig 5.4.1d). At 48 hours, treatment of cells with 1 μ g/ml LPS or 1 μ g/ml LPS with 150 μ M BADGE did not induce TNF α production (37.59, 4.51 ng/mg protein respectively) (fig 5.4.1d).

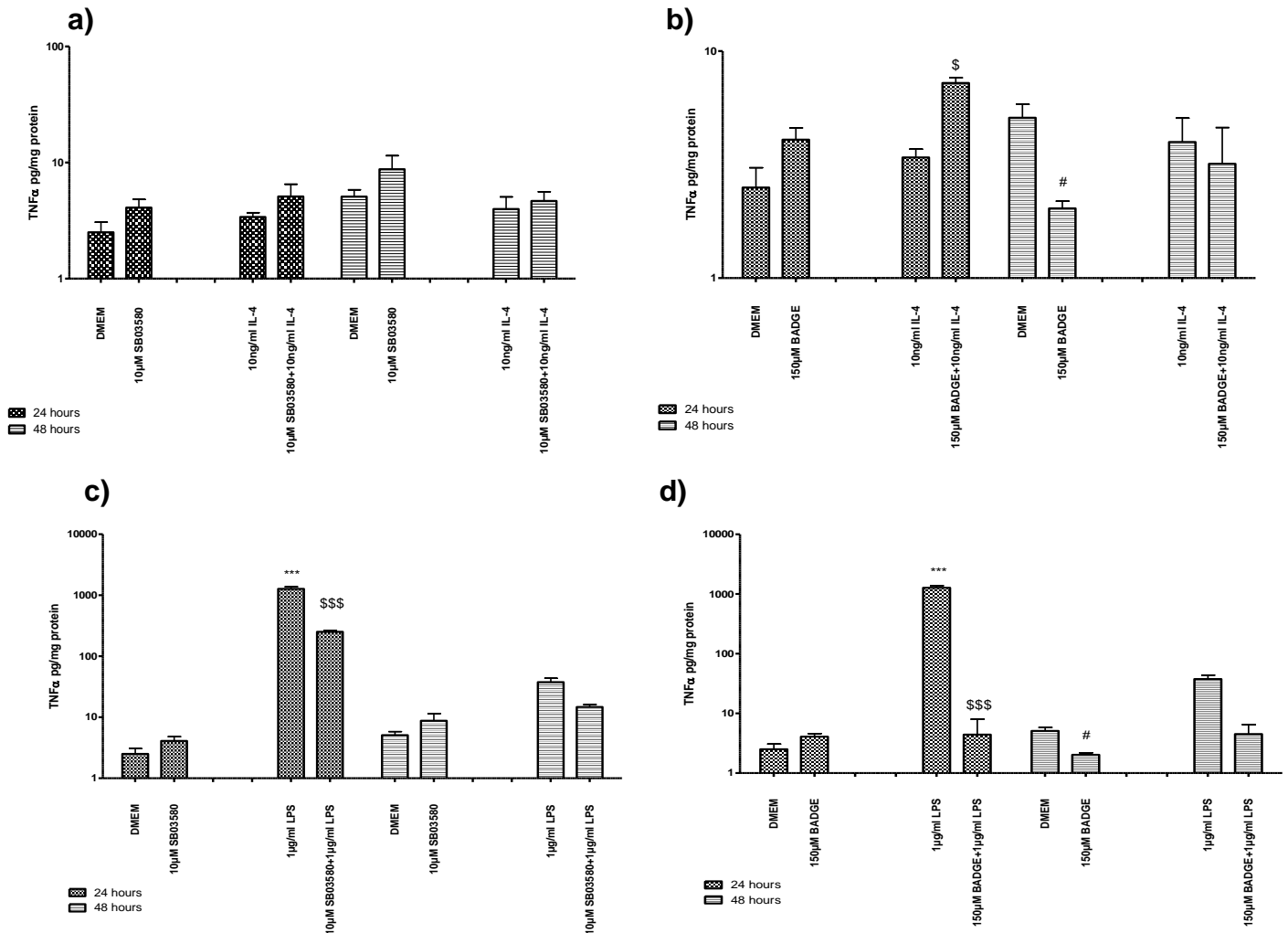


Figure 5.4.1: IL-4 did not induce TNFα in macrophage J774.2 cells. The addition of a) SB203580 did not impact this however, at 24 hours, b) BADGE increased TNFα. LPS required c) p38 and d) PPARγ to produce TNFα 24 hours.

Cells were treated with SB203580 or BADGE for 1 hour before adding 10ng/ml IL-4 or 1μg/ml LPS. Following 24 and 48-hour stimulations, the supernatant was assayed for either IL-10 or TNFα. Unstimulated cells and cells treated with only SB203580 or BADGE were the negative controls. Cells treated with 10ng/ml IL-4 or 1μg/ml LPS were the positive controls. All bars represent the effect of either SB203580 or BADGE on TNFα production by IL-4 and LPS.

The data shown were obtained from 3 independent experiments (n=3). TNFα levels produced following treatment of cells with IL-4 and LPS were statistically compared to the negative control via the One Way ANOVA; as represented by *. Cells treated with SB203580 and BADGE were compared to the positive control with One Way ANOVA; represented by \$. The One Way ANOVA was also used to compare the differences in TNFα production between the unstimulated cells and cells where SB203580 or BADGE were added; represented by #. Data are expressed as mean+/- SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, \$p<0.05, \$\$p<0.01, \$\$\$p<0.001., #p<0.05, ##p<0.01, ###p<0.001.

5.5 Suppression of cytokine release by COX inhibitor

Indomethacin is a non-selective COX inhibitor. Indomethacin, although a potent inhibitor of COX-1, inhibited COX-2 ($IC_{50}=0.48\mu M$) (Blanco et al., 1999, Warner et al., 1999). Indomethacin was found to exacerbate inflammation when administered at 48 hours *in vivo* (Gilroy et al., 1999). Clinical cases have been reported where patients who took indomethacin experienced adverse effects, typically relating to the skin, CNS and gastro-intestinal tract (Boardman and Hart, 1967). As indomethacin has been used in the treatment of inflammatory diseases, the effect of these drugs on the cytokine profile following co-treatment with LPS and IL-4 would be interesting to look at. Inhibition of pro-inflammatory cytokines, would suggest indomethacin to be useful in chronic disease, however inhibition of IL-4 induced anti-inflammatory cytokines would suggest administration of this drug to be harmful, as it impedes resolution.

The addition of 10ng/ml IL-4 to the cells showed an upsurge in the amount of IL-10 produced (102.48 ng/mg protein; $p<0.001^{***}$) at 48 hours compared to the negative control. Indomethacin on its own showed no effect on IL-10 levels compared to the negative control (fig 5.5.1a). When macrophages were co-treated with IL-4 and indomethacin, there was complete inhibition of IL-10 production (undetectable IL-10 levels; $p<0.001^{$$$}$, undetectable IL-10 levels; $p<0.001^{$$$}$, undetectable IL-10 levels; $p<0.001^{$$$}$), (fig 5.5.1a). A similar pattern was reported at 48 hours where there was no IL-10 produced in the negative control. Indomethacin on its own produced no detectable IL-10 (fig 5.5.1a). Addition of IL-4 caused a significant increase in IL-10 (76.68 ng/mg protein; $p<0.001^{***}$) but this was completely blocked in the presence of increasing concentrations of indomethacin (undetectable IL-10 levels; $p<0.001^{$$$}$, undetectable IL-10 levels; $p<0.001^{$$$}$, undetectable IL-10 levels; $p<0.001^{$$$}$).

Upon addition of 1 µg/ml LPS, there was a significant increase in TNFα production (1274.59 ng/mg protein; $p < 0.001^{***}$) in comparison to the negative control (2.51 ng/mg protein). Low concentrations of indomethacin did not effect basal TNFα (2.95 ng/mg protein), but higher concentrations of indomethacin inhibited TNFα (undetectable TNFα levels; $p < 0.05^{\#}$, undetectable TNFα levels; $p < 0.05^{\#}$ ng/mg protein) at 24 hours (fig 5.5.1b). When indomethacin was added to LPS, the levels of TNFα was reduced significantly (123.75 ng/mg protein; $p < 0.001^{$$$}$, 107.13 ng/mg protein; $p < 0.001^{$$$}$, 5.56 ng/mg protein; $p < 0.001^{$$$}$ ng/mg protein); though this did not reach basal levels (fig 5.5.1b). At 48 hours, 1 µg/ml LPS caused a significant increase in TNFα (37.59 ng/mg protein; $p < 0.01^{**}$) (fig 5.5.1b). Similar to 24 hours, when increasing concentrations of indomethacin was added to LPS, the levels of TNFα decreased significantly (16.44 ng/mg protein; $p < 0.05^{\$}$, 18.95 ng/mg protein; $p < 0.05^{\$}$, 7.36 ng/mg protein; $p < 0.001^{$$$}$) (fig 5.5.1b). Low concentrations of indomethacin on its own did not effect basal levels of TNFα (7.52, 3.86 ng/mg protein). Indomethacin of highest concentration blocked basal TNFα production (undetectable TNFα levels ng/mg protein; $p < 0.01^{##}$)

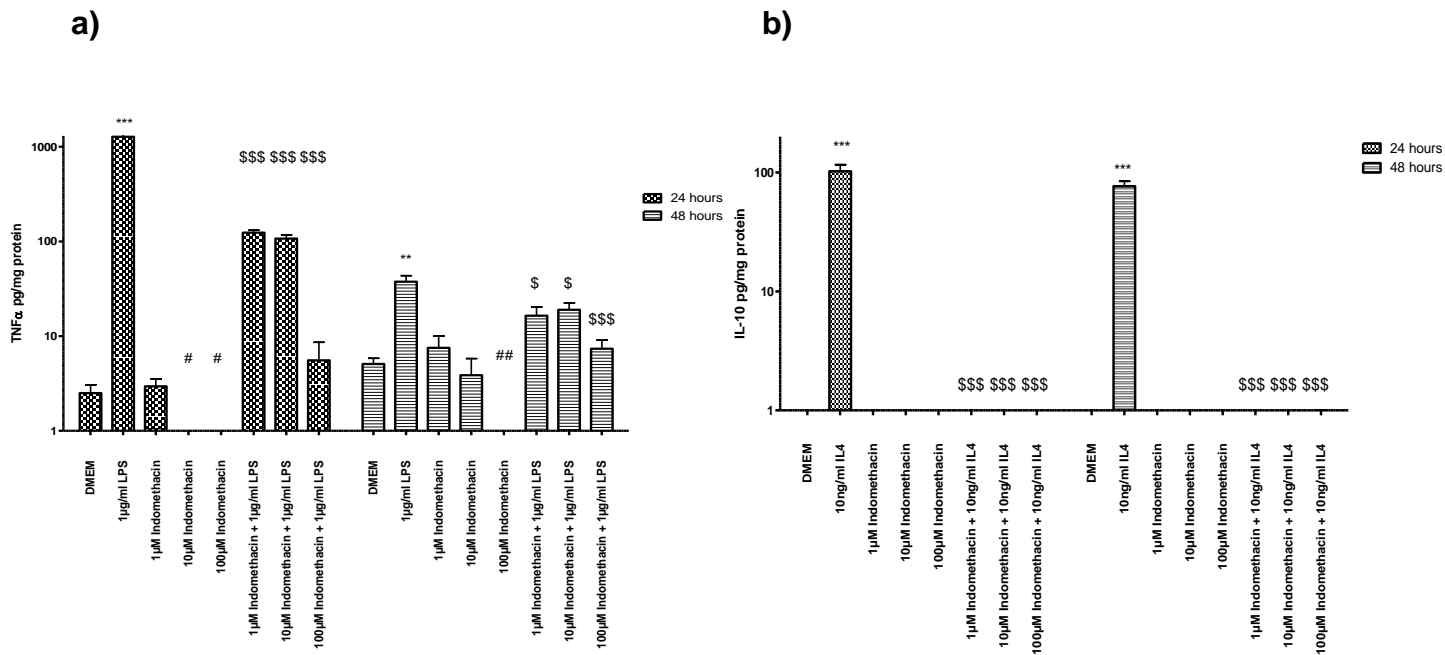


Figure 5.5.1: Indomethacin of increasing concentrations blocked a) IL-10 produced by IL-4 and reduced b) TNFα produced by LPS.

Cells were treated with increasing concentrations of indomethacin for 1 hour before adding 10ng/ml IL-4 or 1μg/ml LPS to the cells for 24 and 48 hours. The supernatant was assayed to measure IL-10 and TNFα. Unstimulated cells and cells treated with indomethacin alone were the negative controls. Cells treated with 10ng/ml IL-4 or 1μg/ml LPS were the positive controls. All bars represent the effect of indomethacin on IL-10 and TNFα produced by IL-4 and LPS respectively.

The data shown were obtained from 3 independent experiments (n=3). One Way ANOVA with the Dunnett's post hoc test was used to compare TNFα and IL-10 levels produced following treatment of cells with IL-4 and LPS compared to unstimulated cells, as represented by *, Cells treated with indomethacin and the positive control, as represented by \$ and between the unstimulated cells and cells treated with only indomethacin; represented by #. Data are expressed as mean+/- SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, \$p<0.05, \$\$p<0.01, \$\$\$p<0.001., #p<0.05, ##p<0.01, ###p<0.001.

5.6 Discussion to results chapter 5

Macrophage polarisation following treatment of cells with LPS and IL-4

5.6.1 Cytokine profile following treatment of cells with LPS and IL-4

The first chapter suggests differential properties for the LPS and diclofenac/IL-4 induced COX-2. As the work in this project is carried out on a macrophage cell line, it would be important to look at the phenotypic state in each stimulation. Similar to the T helper cell dichotomy of cell polarisation (Th1 and Th2 cells), macrophages also have 2 different phenotypes: M1 and M2 macrophages (see 1.2), however there has been cases of heterogeneity amongst these macrophages (see for review: (Geissmann et al., 2010, Gordon and Pluddemann, 2017)). The polarisation of macrophages has been suggested to be driven by the microenvironment (Bystrom et al., 2008) (see for reviews: (Italiani and Boraschi, 2014, Van Overmeire et al., 2014)) which allows these cells to mount a spectrum of functional responses. Abundant M2 stimuli and lack of M1 signals drive preferential polarisation towards M2. Typically, the macrophage phenotypic state is denoted by the Arg1: iNOS ratio (Corraliza et al., 1995). M1 and M2 markers, production of cytokines and activation of specific transcription factors all play a role in identifying the macrophage phenotype (see 1.2)

It was previously shown that IL-4, but not LPS, increased cAMP (fig 4.2.1e, f). cAMP has been associated with an anti-inflammatory phenotype of the macrophage based on the release of IL-10, an anti-inflammatory cytokine and a decline in TNF α , a pro-inflammatory cytokine (Bystrom et al., 2008). AnxA1 was also found to exert pro-resolving downstream effects of cAMP. The cAMP agonists were found to have opposing functions to LPS mediated neutrophil apoptotic effects (Lima et al., 2017);

suggesting cAMP and LPS to be part of two opposing pathways. Thus, the findings made in the previous chapter suggested IL-4 to force macrophages towards an anti-inflammatory phenotype, with the LPS forcing macrophages towards an M1 phenotype.

The following speculations, led us to further clarify whether the macrophage induced by LPS and/or IL-4, which express COX-2, produce pro-/ or anti-inflammatory cytokines. As previously mentioned, cytokine profiles have previously been used to determine the phenotype of the immune cell (Cherwinski et al., 1987). To this end, we measured IL-10 and TNF α , the anti-inflammatory and pro-inflammatory cytokine respectively, following each stimulation.

5.6.2 IL-4 treated macrophages produce IL-10 cytokine

We were able to show all concentrations of IL-4 to significantly release IL-10 from as early as 2 hours up until 48 hours (fig 5.2.1). This suggests the effect of IL-4 to be somewhat early. IL-4 induced IL-10 synthesis was something previously noted (Kambayashi et al., 1996). The ability of low concentrations of IL-4 to induce a biological response has previously been noted (see for review: (Mueller et al., 2002)). The fact that the negative control, where cells were left unstimulated had no IL-10, but the addition of IL-4 stimulated IL-10 release (fig 5.2.1), suggest IL-4 to force the macrophage down an anti-inflammatory pathway. The range of IL-10 production is somewhat narrow between all concentrations of IL-4. This could be due to receptor saturation or maximal response of the IL-4 binding to its receptor being reached (see for review: (Mueller et al., 2002)).

As we were able to identify the role of p38 and PPAR γ on COX-2, we aimed to investigate the role of p38 and PPAR γ on IL-10 release following IL-4 treatment. Co-treating cells with 10ng/ml IL-4 and 10 μ M SB203580 reduced and completely blocked IL-10 production at 24 and 48 hours respectively (fig 5.3.1a). SB203580 on its own did not induce IL-10 production. This confirms the role of p38 in IL-4 signalling routes in an anti-inflammatory pathway. We speculate that SB203580 in IL-4 treated cells prevent the polarisation of the macrophages towards an M2 phenotype. It has previously been shown that silencing or pharmacologically blocking p38 blocks the polarisation of macrophages towards M2 (Jimenez-Garcia et al., 2015). As M2 macrophages produce IL-10, this may explain why the production of IL-10 is completely lost in the presence of SB203580. Campbell et al. (2004) showed p38 did not block IL-10 production in RA tissue. The role of p38 may be dependent on the environment, i.e. in RA, the environment is inflammatory however, an IL-4 treated environment will be anti-inflammatory. Moreover, p38 may have a time-dependent role, as it was shown to have differential effects at 24 and 48 hours.

Treatment of cells with BADGE inhibited the IL-4 induced production of IL-10 (fig 5.3.1b). This suggests PPAR γ to be an absolute requirement for the polarisation of macrophages towards an anti-inflammatory cytokine secreting macrophage; similar to its absolute requirement for the induction of COX-2 (fig 4.3.1.2b). PPAR γ has also been associated with anti-inflammatory signalling pathways downstream to IL-4 (Szanto et al., 2010). The presence of a PPAR γ antagonist showed IL-4 inductive anti-inflammatory effects to be lost. (Paintlia et al., 2006). The ability of BADGE to completely block IL-4 induced COX-2 (fig 4.3.1.2b), suggest the process of inducing COX-2 and that of polarisation, may follow a similar pathway. BADGE works by

inhibiting the transcriptional activation of both PPAR γ and RXR α by inhibiting ligand binding to the receptor (Wright et al., 2000).

We have previously shown inhibition of PPAR γ activity induced by IL-4, in the presence of SB203580 (fig 4.4.2b). This reinforces the theory, whereby we suggest p38 to activate PPAR γ , as inhibiting p38 inhibited IL-10 release, similar to PPAR γ antagonism inhibiting IL-10. The effect of p38 inhibition, may therefore be due to the lack of PPAR γ activation.

As hypothesised, we have shown the involvement of both p38 and PPAR γ in both the induction of COX-2 and the production of anti-inflammatory cytokines. It is thought provoking that following 24 hours of treatment with IL-4, the macrophages did not induce COX-2 (fig 3.2.1), cAMP production was not observed (fig 4.2.1e, f) and PPAR γ was not activated (fig 4.4.2a). However, IL-4 treated macrophages developed an anti-inflammatory phenotype at 24 hours (fig 5.2.1). This suggests that the macrophage may polarise prior to the induction of COX-2; as opposed to the induction of COX-2 assisting with driving macrophage polarisation. This differs to other studies which have shown the inhibition of COX-2 to impact polarisation of the macrophage (Na et al., 2013). It may be possible that IL-4 creates a microenvironment that allows the macrophage to polarise to this functional phenotype (see for review: (Italiani and Boraschi, 2014)).

5.6.3 LPS, but not IL-4 transform macrophages to produce TNF α

In unstimulated cells, the levels of TNF α were relatively low. This can be attributed to weak gene transcription of TNF α (Mijatovic et al., 1997). Interestingly,

throughout the 48-hour treatment of cells with 10ng/ml IL-4, there was basal levels of TNF α , similar to the control. The addition of SB203580 had no effect on the TNF α produced (fig 5.4.1a). This differs somewhat to the addition of BADGE where, specifically at 24 hours, addition of the PPAR γ antagonist caused an increase in TNF α (fig 5.4.1b). This suggests that activation of PPAR γ by IL-4 shuts down TNF α production, highlighting the anti-inflammatory pathways downstream to IL-4 treatment. The fact that PPAR γ is required for IL-10 synthesis and TNF α suppression, reiterates the anti-inflammatory role for PPAR γ in macrophages. Furthermore, this confirms that depending on the stimuli, the macrophage polarises towards a pro-inflammatory or anti-inflammatory phenotype.

The addition of IL-4 to the macrophage J774.2 cells did not affect the basal levels of TNF α in the negative control (fig 5.4.1a, b). This suggests the IL-4 not to initiate a pathway to decrease this pro-inflammatory cytokine; rather, it has no such effect on the cytokine. A similar pattern was observed by Gautam et al. (1992) who showed no effect by IL-4 on LPS induced TNF α in macrophages. This differs to work carried out by Mijatovic et al. (1997) who showed both IL-4 and IL-13 to translationally repress LPS induced TNF α . Repression occurs at the UA-rich sequence which is present in the 3'-UTR. This group noted that the TNF α transcript remained stable however production was reduced, therefore, TNF α suppression is mediated at a translational level by IL-4 (Mijatovic et al., 1997). Seventy two percent inhibition of TNF α was noted via ELISA when the cells were co-treated with 10ng/ml LPS and 5ng/ml IL-4 in both mouse RAW 264.7 and J774 macrophages (Mijatovic et al., 1997). It was suggested that IL-4 may disrupt translation of TNF α by blocking the protein complex which allows the binding to the UA-rich sequence of TNF α mRNA following

treatment of the macrophage with LPS (Mijatovic et al., 1997). Similarly, in microglial cells, 10ng/ml IL-4 induced an M2 macrophage whilst modestly reducing TNF α that was produced by LPS (Ghosh et al., 2016). IL-4 mediated inhibitory effects were not observed in STAT6 null BMDM but repression was seen in wildtype cells (Levings and Schrader, 1999), thus these effects may be driven via STAT6.

Kambayashi et al. (1996) found that the inhibitory effect of IL-4 may be partially mediated via IL-10 which was shown to be increased during co-treatment with LPS. Neutralising IL-10 reversed this IL-4 induced TNF α inhibitory effect (Kambayashi et al., 1996). Similarly, Levings and Schrader (1999) found IL-10 to block TNF α production in peritoneal exudate macrophages (PEC) and BMDM, independent of STAT6, while Chan et al. (2012) showed IL-10 to block TNF α mRNA expression in a STAT3 dependent manner. Chan et al. (2012) showed a 45-minute LPS stimulation followed by a 15-minute stimulation with IL-10 to shift TNF α mRNA from polysomes to monosomes which reduce TNF α protein synthesis. Levings and Schrader (1999) reported IL-4 not to have any effect on TNF α produced by LPS in PEC. Our work did not look at the co-treatment of a pro- and anti-inflammatory stimuli as we were interested in differential expression of COX-2 in comparative studies. None the less, it would be interesting to look at the effect of introducing IL-4 to cells that were treated with LPS and measure cytokine levels.

LPS treated cells showed an upsurge in TNF α levels at 24 hours and this was sustained till 48 hours. Whereas blocking p38 at 24 hours, significantly reduced TNF α production, at 48 hours, blocking p38 had no effect (fig 5.4.1c). Tyrosine phosphorylation of MAPK has been associated with TNF α synthesis following LPS

stimulations (Mijatovic et al., 1997). Sabio and Davis (2014) reviewed p38 α but not p38 β to play a key role in controlling TNF α production. TNF α stability and the initiation of translation were found to be regulated at the level of p38 α . Chan et al. (2012) reported p38 as a requirement for TNF α synthesis as inhibition of this leads to reduced polysome association which ultimately leads to reduced TNF α . It has previously been shown that LPS induced TNF α via NF κ B directed gene transcription with a 10,000-fold increase in its biosynthesis (Mijatovic et al., 1997). It was suggested that p38 may function via the activation of NF κ B by possibly phosphorylating the p65 subunit. SB203580 had no effect on the degradation of I κ B induced by LPS, but luciferase reporter gene assays showed this compound to cause approximately 65% inhibition of NF κ B transcription (Campbell et al., 2004). There may be some cell specificity in the effect of SB203580 because in HeLa and Jurkat cells, this compound did not block TNF α induced gene activity downstream to NF κ B activation (Campbell et al., 2004). Although p38 showed an increase in NF κ B, p38 can also inhibit NF κ B activity following exposure to TNF α . Thus, p38 may play a dual role, whereby it activates TNF α transcription and via an autoregulatory machinery, limits TNF α expression (see for review: (Sabio and Davis, 2014)). It may therefore be conceptual that p38 behaves as a regulator during the inflammatory process

The presence of BADGE significantly reduced TNF α produced by LPS at 24 hours, suggesting a role for PPAR γ in TNF α synthesis (fig 5.4.1d). It is ambiguous as to how LPS significantly reduced PPAR γ activity at 24 hours (fig 4.4.1) but required PPAR γ for the synthesis of TNF α at the same time (fig 4.4.1d). It is possible that LPS did not activate PPAR γ activity as it activates NF κ B (Mijatovic et al., 1997, Campbell et al., 2004). In the absence of BADGE, low levels of cAMP were seen (fig 4.4.2.1b)

with high levels of TNF α (fig 5.4.1d). Co-treatment of LPS and BADGE at 24 hours increased cAMP production (fig 4.4.2.1b). This may coincide with reduced TNF α reported following PPAR γ antagonism (fig 5.4.1d). We speculate the cAMP to work in an opposing manner to TNF α production. Following LPS injection in mice, concentration increases of the PDE inhibitor, CP-80,633, resulted in enhanced cAMP and reduced systemic TNF α (Cheng et al., 1997). Wall et al. (2009) showed cAMP mediated effects on TNF α production in RAW 264.7 macrophages to be dependent on PKA. Treatment of cells with 8Br-cAMP, the cAMP analog, showed robust inhibition of TNF α along with enhanced IL-10 (Wall et al., 2009). This group showed p105 phosphorylation to mediate cAMP dependent inhibition of LPS induced TNF α ; thus, modulating NF κ B (Wall et al., 2009). The C-terminal region of p105 contained a PKA phosphorylation site. cAMP was capable of slowing the nuclear localisation of the p50 and p65 subunits following LPS stimulation (Wall et al., 2009). Therefore, it may be that BADGE induced increase in cAMP that blocked the production of TNF α . Although BADGE impacts cAMP, it does not seem to impact LPS induced COX-2 activity (fig 4.3.1.3). It would be interesting to note whether co-stimulation of LPS and BADGE impacts TNF α levels.

5.6.4 Inhibition of cytokine production by Indomethacin

FACS data showed etodolac, a selective COX-2 inhibitor, to reduce IL-10 in macrophages and increase MHC Class II, CD80 and CD86. Thus, this group suggested COX-2 to be a requirement for the polarisation of macrophages towards the M2 phenotype (Na et al., 2013); highlighting its importance in anti-inflammatory macrophages. Carboxylic acid containing indomethacin has been recognised as a pan COX inhibitor. It was found that the indomethacin derivative compounds, amides and

esters, bind to COX-2. A prototypical compound of this had an IC(50) value of 0.06 μ M for purified human COX-2 (Rommel et al., 2004). If the COX-2 protein induced by both LPS and IL-4 are in fact different, it may perhaps be that they are differentially inhibited by indomethacin, as we have previously seen differential acetaminophen inhibition (fig 3.3.1).

5.6.5 Indomethacin inhibitory effect on IL-10

This leads to the next set of experiments which showed indomethacin treatment in IL-4 treated macrophages to lead to complete inhibition of IL-10 production (fig 5.5.1a). As a drug used in the treatment of chronic infection, it would not be expected that this NSAID would block production of an anti-inflammatory cytokine as that would impede resolution. Previously, indomethacin was found to reduce Treg cells that were induced by antigens (Tonby et al., 2016). Treg cells produce both TGF β and IL-10, both anti-inflammatory cytokines (see for review: (So et al., 2015)). The reduced IL-10 in the presence of indomethacin (fig 5.5.1a) may be due to a similar effect in the macrophage J774.2 cells.

5.6.6 Indomethacin inhibitory effect on TNF α

Indomethacin was shown to reduce LPS induced TNF α as expected (fig 5.5.1b). Previously, it was shown that indomethacin can reverse the TNF α mediated effects on permeability of the endothelial cell monolayer (Mark et al., 2001). Some of these effects were attributed to the inhibition of PGE₂ which was found to be stimulated by TNF α (Mark et al., 2001). A more recent study using C2C12 tumour cells showed TNF α to increase COX-2 and PGE₂ synthesis, which was capable of mediating some TNF α effects (Park et al., 2017). Thus, indomethacin blockage of PGE₂ may explain

the reduced TNF α (fig 5.5.1b). A similar observation was made by Zheng et al. (2017) who reported indomethacin to downregulate levels of COX-2. TNF α mediated nociception was partially lost in the presence of indomethacin, thus reinforcing the opposing effect of indomethacin on this pro-inflammatory cytokine (Verri et al., 2007).

This differs to findings made by Teeling et al. (2010) who showed that although indomethacin reversed LPS induced behavioural effects in mice, it had no effect on TNF α levels. Whereas LPS co-treated with DEX reduced TNF α , LPS with indomethacin increased TNF α (Teeling et al., 2010). A similar pattern was observed by Hartel et al. (2004) who showed LPS induced TNF α production in a directly proportional manner from concentrations ranging 1 μ M to 50 μ M of LPS. The range of concentrations of indomethacin used in this study may be too high, hence the forced inhibition (fig 5.5.1). Indomethacin has been shown to activate PPAR γ by direct binding (Lehmann et al., 1997). Increased PPAR γ correlated with reduced TNF α (Kajita et al., 2004). This further complicates the findings observed in the experiment (fig 3.3.4).

The fact that indomethacin blocked the production of the anti-inflammatory cytokine, we suggest that this drug may prevent the polarisation of the macrophage towards the anti-inflammatory phenotype via inhibition of COX-2. The effect of COX-2 inhibition by indomethacin on both cytokines, suggest the COX-2 induced by LPS and IL-4 may share some similarity in structure that enable common inhibition. To further understand this, the impact of GC should be investigated in regards to the expression of COX-2 and macrophage polarisation.

5.7 Chapter conclusion

It is evident IL-4 transformed the macrophages down the anti-inflammatory pathway while LPS drove macrophages down the pro-inflammatory pathway. This is due to the production of IL-10 following IL-4 treatment (fig 5.2.1) and TNF α production following LPS treatment. The requirement for both p38 (fig 5.3.1a) and PPAR γ (fig 5.3.1b) in the production of IL-10 by IL-4 further confirmed a similar signalling pathway for both COX-2 (4.6) and IL-10 production. The fact that indomethacin inhibited cytokine production induced by both stimulants (fig 5.5.1) provided controversy in its administration. Indomethacin blocking IL-10 production, suggests a relationship between COX-2 and the phenotype of the macrophage. COX-2 may sustain the polarisation state of the macrophage due to the PG it produces. Thus, inhibition of this, may revert the macrophage to its state pre-stimulation.

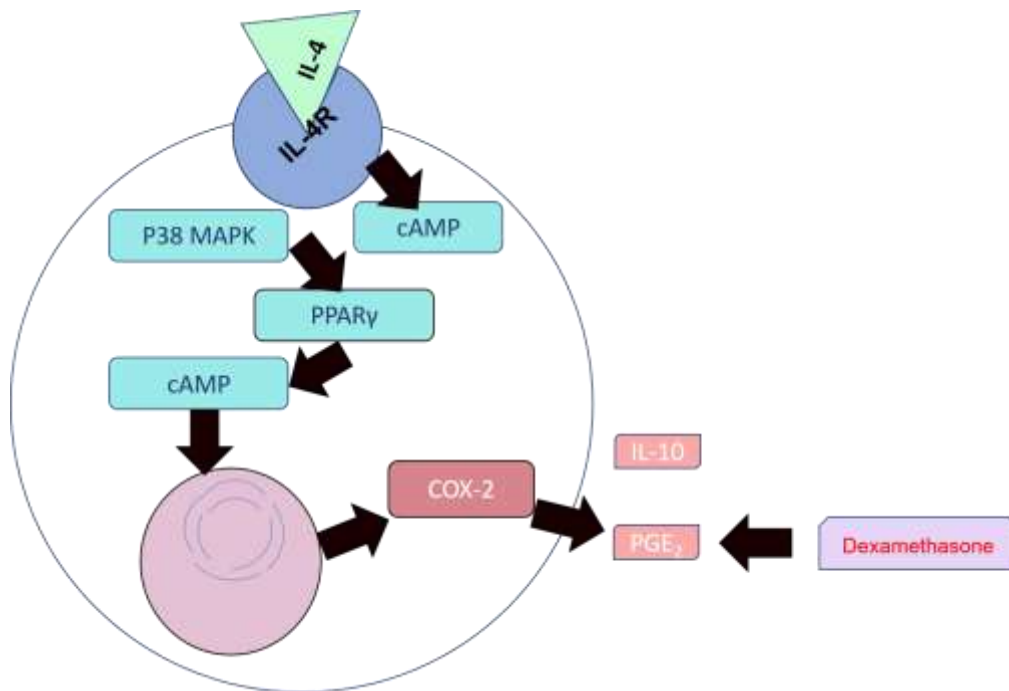


Figure 5.7.1 *Diagram to conclude chapter*

Macrophage J774.2 cells that were treated with IL-4 produced IL-10 anti-inflammatory cytokines. This production is dependent on both p38 and PPAR γ because inhibiting these with SB203580 and BADGE, blocked IL-10 secretion. Co-treating the J774.2 macrophages with increasing concentrations of indomethacin blocked IL-10 production completely.

Chapter 6: Effect of dexamethasone in macrophages stimulated with LPS, diclofenac and IL-4

6.1 Antagonistic relationship between dexamethasone and LPS

DEX is a GC known to induce anti-inflammatory pathways. This has been shown with the reduction of pro-inflammatory markers such as CD40, CD80 and CD86 and production of anti-inflammatory cytokines such as IL-10 (Xia et al., 2005). DEX has been characterised as a regulator of COX expression and activity in the inflammatory response (Masferrer et al., 1992). The antagonistic effects of LPS and DEX has been documented in human blood monocytes. COX-2 mRNA induced by IL-4 was inhibited by DEX ($IC_{50}=0.0073\mu M$). Peskar (2001) found intragastric acid to induce COX-2 mRNA and this was inhibited by DEX without effecting COX-1 mRNA (Peskar, 2001). LPS was shown to induce COX-2 in the brain and both $1\mu M/L$ DEX and $100\mu M/L$ NS-398, the selective COX-2 inhibitor, reduced COX-2 expression and PGE_2 production ex vivo in LPS treated leptomeningeal tissue (Brian et al., 1998). Thus, the antagonistic effects of LPS and DEX has been well documented. It was suggested that DEX exerts its effects by inhibiting PLA_2 (Sampey et al., 2000) or destabilising COX-2 mRNA via p38 inhibition (Barrios-Rodiles and Chadee, 1998).

6.2 Effect of dexamethasone on COX-2 induction by LPS, diclofenac and IL-4

We were interested in investigating whether DEX differentially impacts COX-2 expression depending on the inducer. To further characterise the LPS, diclofenac and IL-4 induced COX-2 and assess whether they are differentially inhibited by DEX, the cells were pre-treated with DEX for 1 hour prior to the addition of the stimulant. Cells were either left untreated (negative control), or treated with 0.1% ethanol (vehicle

control), 1µg/ml LPS, 500µM diclofenac or 10ng/ml IL-4 alone (positive control) and the stimulants: 1µM, 2µM, 5µM and 10µM DEX with 1µg/ml LPS, 500µM diclofenac and 10ng/ml IL-4. At the end of the 24 and 48-hour stimulations, the protein content was assayed using Bradford assay following cell lysis (see 2.3). For Western blot analysis, (see 2.4), 20µg protein was loaded on the gel. Anti-COX-2 antibodies were added to PVDF membranes to observe COX-2 protein expression (see 2.10).

The negative and vehicle control showed no COX-2 expression as expected. Increasing concentrations of DEX on its own did not induce COX-2 expression at both 24 and 48 hours (fig 6.2.1). When the macrophages were treated with LPS, COX-2 expression was induced. Pre-treating cells with low concentrations of DEX and LPS showed a reduction in the intensity of COX-2 expression (fig 6.2.1a). With greater concentrations of DEX, the intensity of the COX-2 bands increased. The same was noted at 48 hours, where 1µM and 2µM DEX caused a reduction in LPS induced COX-2 but 5µM and 10µM DEX increased the expression of COX-2 (fig 6.2.1b). COX-2 was induced by 500µM diclofenac and 10ng/ml IL-4 at 48 hours. The addition of DEX reduced this expression at low concentrations, but 5 and 10µM DEX completely abolished diclofenac induced COX-2 (fig 6.2.1c). IL-4 induced COX-2 was reduced by high concentrations of DEX (fig 6.2.1d).

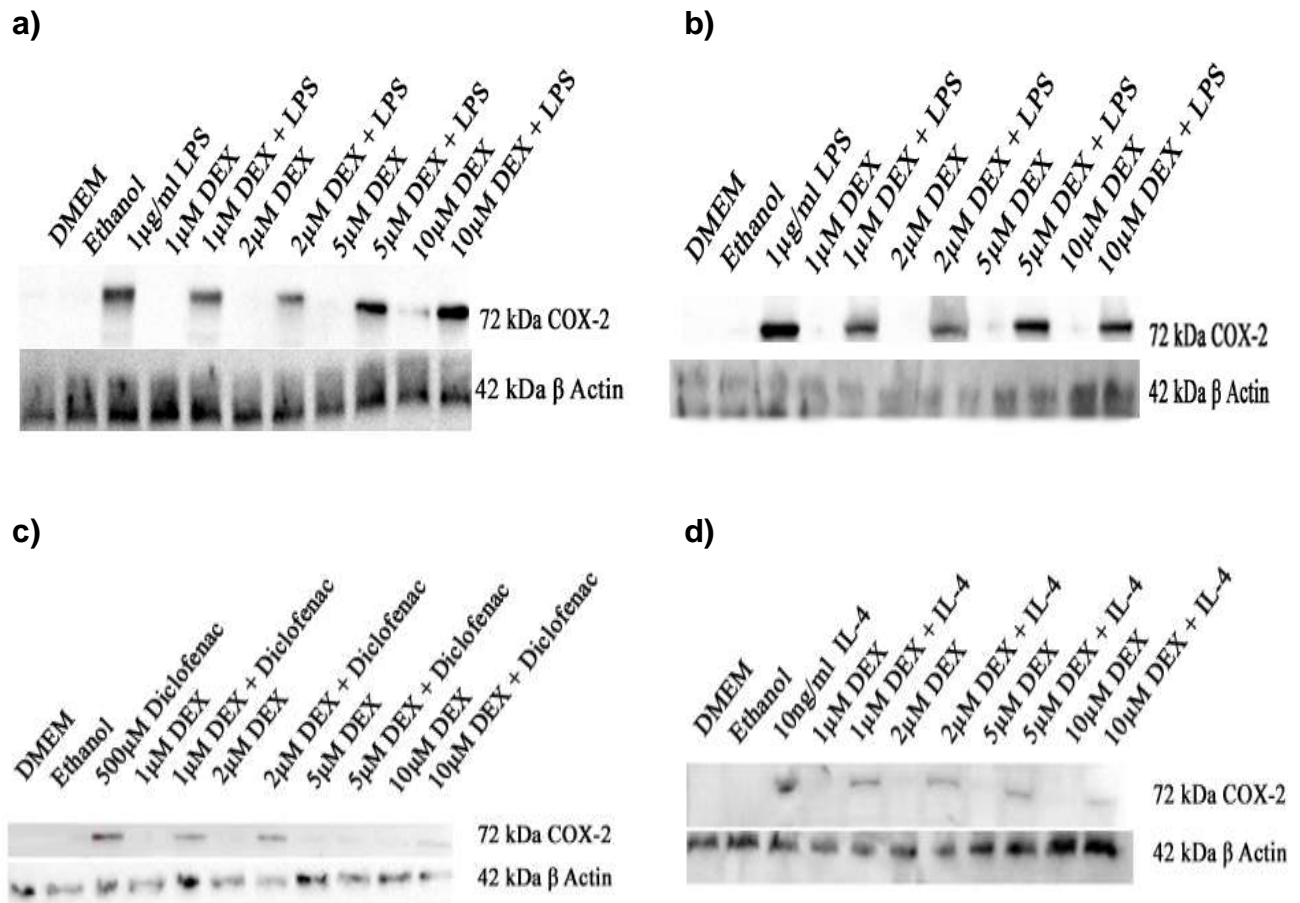


Figure 6.2.1: Low DEX concentrations reduced while high DEX concentrations increased LPS induced COX-2 expression respectively at a) 24 and b) 48 hours. High concentrations of DEX reduced c) diclofenac induced COX-2 while DEX reduced IL-4 induced COX-2 expression.

Cells were treated for 1 hour with increasing concentrations of DEX before 1 μ g/ml LPS, 500 μ M diclofenac or 10 ng/ml IL-4 was added to the cells for 24 and 48 hours. Anti-COX-2 and anti- β -Actin antibodies were added to the membrane in western blot experiments. Unstimulated cells and cells treated with DEX alone were the negative controls. The vehicle control was cells treated with ethanol. The positive controls consisted of cells treated with LPS, diclofenac and IL-4 alone. The data shown were obtained from 3 independent experiments ($n=3$).

6.3 Dexamethasone inhibition of cytokine release by LPS and IL-4 in macrophages

Considering indomethacin had inhibitory effects on both the production of TNF α and IL-10, we sought to investigate the effect of DEX on the macrophage phenotype. As GC are anti-inflammatory drugs, it is expected that they would give rise to IL-10 production whilst reducing TNF α . DEX functions by binding GR which has been shown to reduce expression of PPAR γ (Inoue et al., 2000). As PPAR γ inhibition was shown to inhibit production of IL-10 by IL-4 (fig 3.3.3), the effect of DEX would be quite intriguing in this experiment.

Supernatants were assayed for TNF α and IL-10 content using ELISA techniques (see 2.8). The following experiment was only carried out on cells treated with LPS or IL-4 co-treated with 1, 2 and 5 μ M DEX. Ten micromolar DEX was not used because the effect of 5 and 10 μ M DEX appear to be similar. All readings were normalised to protein concentration.

Treating cells with 10ng/ml IL-4 caused cells to produce IL-10 (102.48 ng/mg protein; $p < 0.001^{***}$). All concentrations of DEX caused an increase in basal IL-10 (13.74 ng/mg protein; $p < 0.001^{###}$, 14.3 ng/mg protein; $p < 0.001^{###}$, 24.46 ng/mg protein; $p < 0.001^{###}$ respectively) compared to the negative control (undetectable levels of IL-10). Co-treating cells with 10ng/ml IL-4 and 1 μ M DEX showed a significant decrease in IL-10 production (34.13 $p < 0.01^{\$}$ ng/mg protein) (fig 6.3.1a). Both 2 and 5 μ M DEX had no effect on IL-4 induced IL-10 production at 24 hours (73.18, 138.61 ng/mg protein) (fig 6.3.1a) .

At 48 hours, IL-4 treatment of cells caused a significant increase in IL-10 production (76.68 ng/mg protein $p < 0.001^{***}$). DEX of all concentrations significantly increased IL-10 production (36.76 $p < 0.001^{###}$, 54.92 $p < 0.001^{###}$, 54.22 $p < 0.001^{###}$ ng/mg protein) compared to the negative control (fig 6.3.1a). The addition of DEX to IL-4 had no effect on IL-10 produced by the macrophages (37.73, 81.79, 78.68 ng/mg protein) (fig 6.3.1a) compared to cells treated with IL-4 alone.

Increasing the concentration of DEX showed low to no TNF α production by the macrophages (6.24, 0 $p < 0.05^{\#}$, 0 $p < 0.01^{##}$ ng/mg protein) (fig 6.3.1b). LPS significantly increased TNF α production (1274.59 ng/mg protein $p < 0.001^{***}$). Co-treating cells with LPS and DEX significantly reduced TNF α levels produced by the macrophages (380.01 $p < 0.01^{\$}$, 300.11 $p < 0.001^{$$$}$, 11.4 $p < 0.001^{$$$}$ ng/mg protein) (fig 6.3.1b). At 48 hours, 1 μ M DEX did not effect basal TNF α but higher DEX concentrations blocked TNF α produced by the macrophages (10.28 $p < 0.001^{###}$, 0 $p < 0.001^{###}$, 0 $p < 0.001^{###}$ ng/mg protein) (fig 6.3.1b). Adding DEX to the LPS treated cells significantly reduced TNF α produced; with higher concentrations of DEX completely blocking TNF α production (11.22 $p < 0.05^{\$}$, 0 $p < 0.001^{$$$}$, 0 $p < 0.001^{$$$}$ ng/mg protein) (fig 6.3.1b).

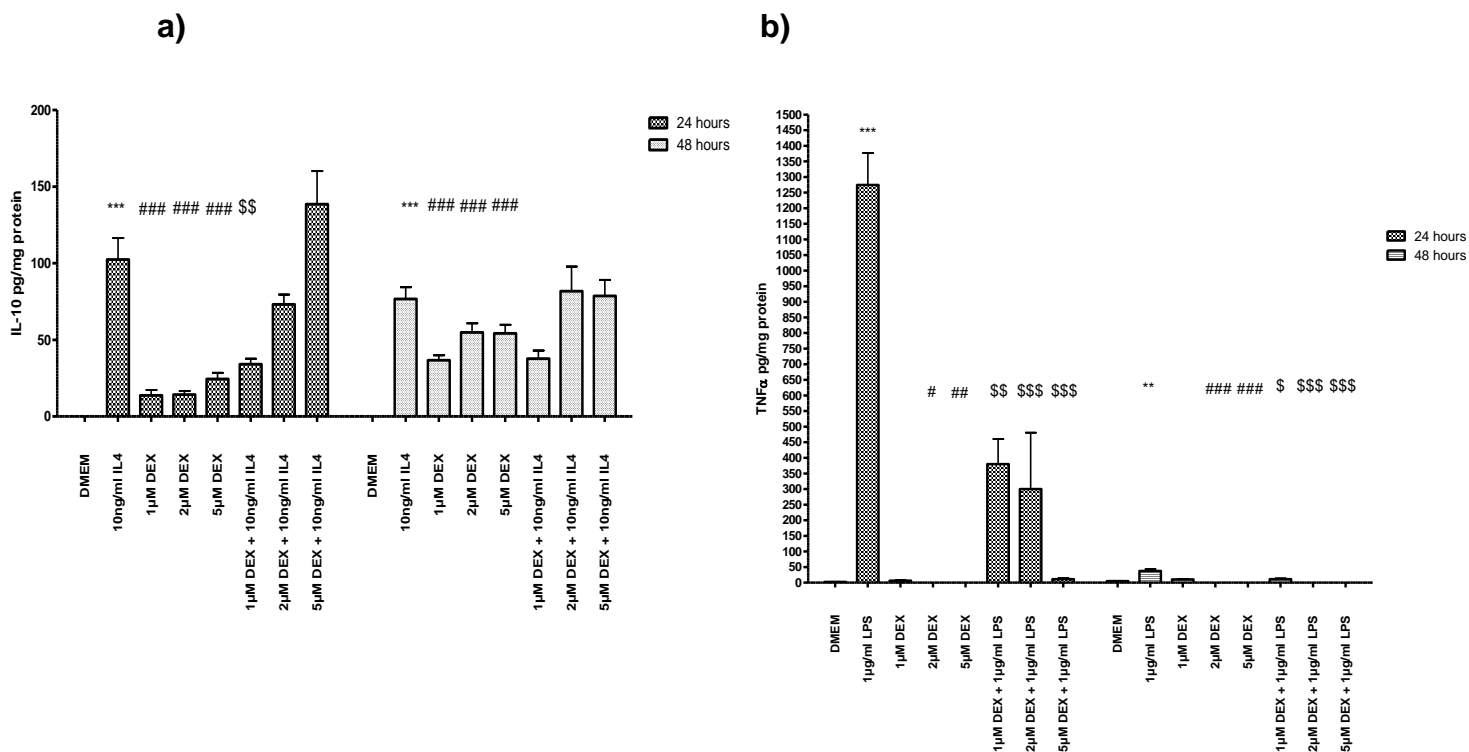


Figure 6.3.1: DEX induced IL-10 production and had no additive effect on a) IL-4 induced IL-10. DEX reduced b) LPS induced TNFα production at both 24 and 48 hours.

Cells were treated with increasing concentrations of DEX for 1 hour before adding 10ng/ml IL-4 or 1μg/ml LPS to the cells. Following 24 and 48-hour stimulations, the supernatant was assayed for PGE₂ and this was standardised to the protein concentration. Unstimulated cells and cells treated with DEX alone were the negative controls. Cells treated with 10ng/ml IL-4 or 1μg/ml LPS were the positive controls. All bars represent the effect of DEX on IL-10 and TNFα produced by IL-4 and LPS respectively.

The data shown were obtained from 3 independent experiments (n=3). IL-10 and TNFα levels produced following treatment of cells with IL-4 and LPS were statistically compared to the negative control via the One Way Anova with the Dunnett's post hoc test; as represented by *. Cells treated with DEX were compared to the positive control with the One Way ANOVA and the Dunnett's post hoc test; represented by \$. The same test was used to compare the differences in cytokine production between the unstimulated cells and cells treated with only DEX; represented by #. Error bars are SEM; *p<0.05, **p<0.01, ***p<0.001.

6.4 Discussion to results chapter 6

The role and effect of dexamethasone on IL-4 treated cells

6.4.1 Effect of dexamethasone on COX-2 expression

DEX is a GC with anti-inflammatory properties. Typically, DEX has been reported to repress production of anti-inflammatory cytokines by interfering with the gene transcription of pro-inflammatory genes (see for review: (Coutinho and Chapman, 2011)). We were able to show low concentrations of DEX to inhibit LPS induced COX-2 but not high concentrations of DEX (fig 6.2.1a, b). The inhibitory effect of DEX on LPS is a phenomenon backdated to the 1990s (Fu et al., 1990, Barrios-Rodiles and Chadee, 1998, Inoue et al., 2000, Yano et al., 2007). Research has shown DEX to have a marginal inhibitory effect on COX-1 with a more profound effect on COX-2 (Masferrer et al., 1990). The antagonistic relation between LPS and DEX has been visited. Fu et al. (1990) showed LPS to elevate COX protein levels and increase PGE₂ AND TXA₂ while DEX inhibits LPS induced COX-2. Barrios-Rodiles *et al.*, (2015) has shown 1µM DEX to rapidly reduce 100ng/ml LPS induced COX-2.

Inoue et al. (2000) suggest LPS to reduce PPAR_γ expression thus reducing the COX-2 synthesised 15d-PGJ₂ binding to its receptor. At this time, LPS is believed to induce GR expression thus increasing the sensitivity to DEX. It may therefore be possible, that PPAR_γ behaves antagonistically to GR despite both 15d-PGJ₂ and DEX having COX-2 suppressing properties. It has been suggested that DEX possesses the ability to suppress this pro-inflammatory protein through the inhibition of PLA₂ (Sampey et al., 2000), however Fu et al. (1990) showed DEX to have no effect on PLA₂. DEX is believed to destabilise β-globulin COX-2 reporter mRNA by inhibiting

p38. Another paper has specifically shown DEX to act downstream of MKK6 and upstream of MK2 (Lasa et al., 2001).

DEX inhibited diclofenac induced COX-2 (fig 6.2.1c) and IL-4 induced COX-2 expression (fig 6.2.1d) with increasing concentrations. This may be explained by the antagonistic effect of PPAR γ and GR. Diclofenac has been shown to require PPAR γ to induce COX-2 (fig 4.3.1.2a). DEX might reduce PPAR γ expression via the up-regulation of GR (Inoue et al., 2000), thus suppressing COX-2 expression by diclofenac. Comera and Russo-Marie (1995) have shown DEX to cause secretion of AnxA1 in human blood monocytes and peritoneal leukocytes. This is similar to the effect of the cAMP inducing agent, forskolin. cAMP is a requirement for IL-4 induced COX-2 expression (fig 4.1.2.1b). This may explain why IL-4 induced COX-2 expression was never completely abolished by DEX.

Wu et al. (1996) showed that in NIH3T3 cells, DEX induces PPAR γ independent to protein synthesis via GR. This appears to be dependent on C/EBP (Wu et al., 1996). It has been shown that GR inhibit NF κ B transactivation by inducing I κ B expression. This transcription factor has previously been shown to be required for LPS induced COX-2 expression (Ayoub et al., 2009). Chistyakov et al. (2015) gave evidence for the activation of NF κ B to shutdown PPAR γ . This group suggested the TLR/NF κ B to mediate suppression of PPAR γ , thus LPS was found to reduce PPAR γ mRNA as early as 2 hours following stimulations (Chistyakov et al., 2015); something we were able to observe (fig 4.4.1).

6.4.2 Effect of DEX on IL-10 and TNF α production following stimulations

Whereas the lowest concentration of DEX reduced IL-4 induced IL-10 synthesis, all other concentrations of DEX had no effect on IL-4 induced IL-10 at 24 hours. DEX had neither a suppressive nor an additive effect on IL-4 induced IL-10 synthesis (fig 6.3.1a). We have shown cells treated with only DEX to produce IL-10 (fig 6.3.1a) and with increasing concentrations of this, basal TNF α was inhibited (fig 6.3.1b). We found DEX to inhibit production of TNF α in macrophages following LPS treatment (fig 6.3.1b). This has previously been noted in many studies (see for review: (Coutinho and Chapman, 2011)). Thus, DEX appears to drive the macrophages in a single direction. A similar effect was previously reported where, Xia et al. (2005) showed DEX to induce IL-10 production in dendritic cells with reduced IL-12. This was suggested to occur via phosphorylation of ERK. IL-10 and TNF α were both shown to modulate the sensitivity of DEX (Franchimont et al., 1999).

Patel et al. (2012) showed the effects of DEX to be mediated via AnxA1 as, DEX could inhibit pro-inflammatory mediators including TNF α but was unable to exert this effect when AnxA1 was knocked out. Citarella et al. (2009) showed LPS to induce TNF α in PMN leukocytes and this was significantly reduced by both IL-10 and DEX. Similarly, DEX was found to suppress TNF α induced by LPS in wild type BMDM (Abraham et al., 2006). This inhibition was reduced in the absence of DUSP1, a protein involved in p38 inactivation. Overexpression of DUSP1 was found to dampen LPS induced pro-inflammatory responses. Interestingly, this group found DEX to induce DUSP1, thus suggesting, DEX to function by activating DUSP1 mediated p38 inactivation (Abraham et al., 2006).

The use of anti-inflammatory cytokine and GC treatment has commonly been investigated. Citarella et al. (2009) reported DEX and IL-10 to equally block LPS stimulated TNF α and IL-1 β in PMN leukocytes as shown by ELISA. Whereas increasing concentrations of DEX blocked TNF α , lower concentrations appeared to stimulate IL-10 while higher concentrations inhibited IL-10 in LPS treated cells (Franchimont et al., 1999). This suggests a dual role of DEX depending on the concentration and may explain why COX-2 expression was increased following treatment of LPS with 5 and 10 μ M DEX (fig 6.2.1a,b).

The sensitivity to DEX appears to be increased as TNF α concentration was reduced and IL-10 was increased (Franchimont et al., 1999). Whereas up to 48-hour stimulations with either TNF α or IL-10 did not force changes in GR expression, at 48 hours, TNF α and IL-10 forced a reduction and increase in GR concentration, respectively, with no effect on the binding affinity to DEX (Franchimont et al., 1999). This suggests IL-10 produced by IL-4 treated macrophages could possibly mediate inhibitory effects on TNF α via GR. This group suggested IL-10 to work synergistically with GC however, there is no evidence for that in the production of IL-10 when DEX was administered (fig 6.3.1a). Thus, the inhibitory effect of DEX on TNF α (fig 6.3.1b) may be driven by IL-10 induced increased GR sensitivity.

6.5 Chapter conclusion

Different concentrations of DEX have different effects on LPS and IL-4 induced COX-2 expression. Whereas low and high concentrations of DEX reduced and increased LPS induced COX-2 expression (fig 6.2.1a,b), increasing concentrations of DEX reduced IL-4 induced COX-2 expression (fig 6.2.1d). A cytokine profile analysis

showed DEX to sustain IL-4 induced IL-10 production (fig 6.3.1a) but reduced LPS induced TNF α production (fig 6.3.1b). Thus, there is evidence that the IL-4 treated macrophages are transformed into anti-inflammatory macrophages and the effect of IL-4 compared to DEX is somewhat similar in regards to the phenotypic function.

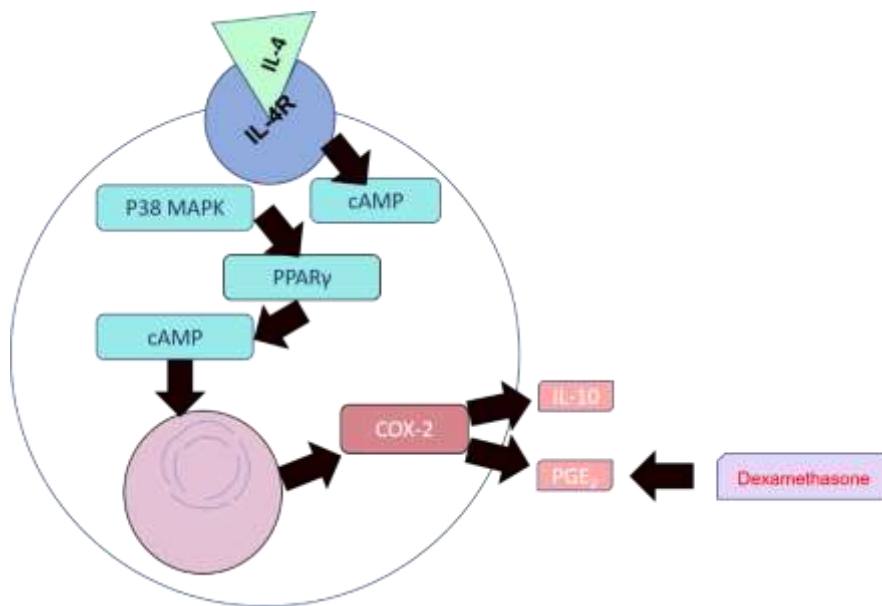


Figure 6.5.1 Diagram to conclude chapter

Introducing increasing concentrations of DEX to macrophage J774.2 cells treated with 10ng/ml IL-4 for 48 hours, reduced COX-2 expression (fig 6.2.1d). Furthermore, 48-hour DEX treatment induced IL-10 production in these macrophages. DEX did not further increase or decrease IL-4 induced IL-10 secretion in this set of macrophages.

7.0 Final conclusion

We aimed to identify a unique signalling pathway that was involved in both IL-4 induced polarisation of macrophages and the induction of a catalytically active COX-2. We hypothesised this pathway to be distinct to the signalling pathway downstream to LPS treated macrophages. Through a series of pharmacological experiments, we were able to identify IL-4, the anti-inflammatory cytokine, as an inducer of COX-2 protein and a stimulator of macrophage polarisation towards an anti-inflammatory phenotype.

To identify the endogenous inducer of the late induced COX-2 that was physiologically relevant, we demonstrated the induction of COX-2 protein at 48 hours by IL-4. This pathway shared significant aspects with the signalling machinery involved in the polarisation of macrophages towards an anti-inflammatory phenotype; in respect to the LPS induced pro-inflammatory macrophages.

The MAPK, p38, the nuclear receptor, PPAR γ and the second messenger, cAMP were shown to be required for the induction of COX-2 by IL-4 in macrophage J774.2 cells. Inhibiting either of these abolished COX-2 expression in this cell line. IL-4 was shown to directly induce PPAR γ activity via p38, specifically following 30 minutes and 48-hour stimulations. The following pharmacological experiments enabled a sequential order of events to be attained; thus, increasing cognizance in COX-2 induction.

We believe that the induction of COX-2 is tightly controlled as there was a delay in its expression and activity depending on the stimuli. Whereas, a PPAR γ agonist, rosiglitazone, could induce COX-2 expression *in vitro* by 24 hours, IL-4 did not induce COX-2 before 48 hours. PPAR γ activity was induced as early as 30 minutes and the expression of p38 in the induction of COX-2 was observed by 24 hours. In addition, LPS was able to induce COX-2 much earlier than IL-4 suggesting differential temporal control depending on the stimulus. We assume this temporal control following IL-4 treatment to be regulated at the level of PPAR γ , p38, cAMP or possibly miR which is a growing area in research.

Interest was shed on the phenotypic nature of the macrophage that expressed COX-2. To understand the phenotypic state of the macrophage within the complex pool of macrophage plasticity and heterogeneity, both cAMP and cytokine levels were measured following each stimulation. High levels of cAMP accompanied with high IL-10 and low TNF α levels were indicative of an anti-inflammatory macrophage phenotype following IL-4 stimulations. This differed to no cAMP, no IL-10 and high TNF α levels following LPS treatment of macrophages. Interestingly, both macrophages expressed COX-2. As it was alleged that COX-2 is an immediate early gene in the pro-inflammatory phase of the inflammatory response, it was particularly interesting that the anti-inflammatory macrophage expressed this protein *in vitro*. We were able to demonstrate the critical role of both p38 and PPAR γ in the production of IL-10 as blocking these completely wiped out IL-10 production at the end of the 48-hour treatment. This draws strong parallel between the pathways involved in COX-2 induction and macrophage polarisation following IL-4 treatment.

Drugs currently in the market may actuate negative side effects due to inhibition of the anti-inflammatory COX-2 protein which we believe to be involved in pro-resolving pathways; however extensive work must be done to conclude this. We demonstrated this concept by the ability of indomethacin to block IL-10 and acetaminophen to inhibit COX-2 activity, that was induced by IL-4 in this cell line. This is clinically relevant, as IL-4 could potentially be the appropriate target in engendering a pro-resolving response during chronic diseases. This thesis puts emphasis on the roles of p38 MAPK, PPAR γ and cAMP in these pathways and so would also require attention in this area of research.

Although a unique signalling pathway was provided for the induction of COX-2 via an anti-inflammatory route, there is yet a lot to assimilate in this process. This project therefore, opens doors for further research in this field.

8.0 Further work

The major limitation of this work is that the experiments were carried out on a cell line: macrophage J774.2 cells and so it would be important to replicate some of the key experiments in this thesis in primary macrophages and/or in *in vivo* models.

To further understand the signalling machinery downstream to IL-4 treatment, antagonising the IL-4R to observe whether IL-4 exerts its effects via this receptor can be confirmed. This would suggest IL-4R as a valid therapeutic target in the drug market to instigate resolution, especially in chronic conditions. Using immunofluorescence or Western blot techniques, whether STAT6 is activated and required in both the induction of COX-2 and polarisation of macrophages can be confirmed. This would be extremely useful as STAT6 is commonly activated and required for downstream anti-inflammatory effects of IL-4. In addition to this, measuring expression of typical phenotypic markers on macrophages following treatment with either LPS or IL-4 would confirm the polarisation state of the macrophage; though this may be challenging considering the heterogeneity in different tissues.

Although we have shown p38 and PPAR γ to be required for the production of IL-10 by IL-4, we have yet to confirm whether cAMP antagonism can block IL-10 production. This thesis has speculated PGE₂ to exert its effects via the EP4 receptor, however in further experiments, this should be confirmed. Furthermore, whether blocking COX-2 blocks p38 phosphorylation, PPAR γ activity and cAMP production would be key to clarify any negative or positive feedback mechanisms in place. This may also bring us closer to understanding the potential anti-inflammatory properties of COX-2.

It would be interesting to measure the dynamic functions of COX-2 following the different stimulations. One such function is wound healing which is associated with resolution and anti-inflammatory responses. Inducing a scratch on a confluent monolayer of cells represents a wound. Closure of the scratch can be observed following addition of the supernatant that was collected from cells treated with either IL-4 or LPS. This supernatant would provide the microenvironment, either pro- or anti-inflammatory, which drives the polarisation of macrophages. Comparing these findings to observations made in the presence of a COX-2 inhibitor will enable conclusions to be drawn in regards to the anti-inflammatory role of COX-2 in macrophages.

Using *in vivo* models of both acute and chronic inflammation, it would be important to assess IL-4 and LPS signalling during resolution and measure various parameters including cytokine production, phosphorylation of p38, activation of PPAR γ , production of cAMP and production of PGE₂. Analysing the expression of anti-inflammatory and pro-inflammatory markers alongside this would enable conclusions to be made in regards to a unique signalling pathway for both the polarisation of macrophages and the induction of COX-2.

This thesis has provided some novel addition to the literature in pathways that link both macrophage polarisation towards an anti-inflammatory phenotype and the induction of COX-2 by IL-4. Additional experiments will therefore greaten this depth of understanding and eventually provide further physiological relevance to these findings.

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10.0 Communications

The requirement of p38-MAPKinase and peroxisome proliferator-activated receptor- γ for the induction of cyclooxygenase-2 by interleukin-4 drives macrophages into an anti-inflammatory phenotype

Poster communication presented on Monday 11th December 2017 at Pharmacology 2017 organised by the British Pharmacological Society at Queen Elizabeth II Conference Centre, Westminster.

Characterisation of The Signalling Mechanisms Involved In The Induction Of Cyclooxygenase-2 By Interleukin 4

Poster communication presented on Tuesday 13th December 2016 at Pharmacology 2016 organised by the British Pharmacological Society at Queen Elizabeth II Conference Centre, Westminster.

Characterisation of a potentially novel cyclooxygenase-2 variant enzyme and its involvement in the resolution phase of inflammation

Oral communication presented on Friday 10th March 2017 at The University of East London.

Characterisation of the potentially anti-inflammatory COX-2 during resolution of inflammation

Oral communication presented on Tuesday 16th February 2016 at The University of East London.

Characterisation of a potentially novel cyclooxygenase-2 variant enzyme and its involvement in the resolution phase of inflammation?

Oral communication presented on Wednesday 11th February 2015 at The University of East London.